Wind- and Bird-mediated Gene Flow in *Pinus cembra*: Effects on Spatial Genetic Structure and Potential Close-relative Inbreeding

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Summary

Various processes influence the genetic composition and diversity of a population, with gene flow within and among populations playing a crucial role. If the exchange of genes is restricted, probabilities for genetic drift and the occurrence of matings among related individuals are elevated. This might lead to increased levels of homozygosity, which was found to have detrimental effects on offspring fitness in various plant and animal species. Future generations may thus be subjected to a loss of genetic diversity and, hence, exhibit reduced adaptive potential in the long term. Therefore, detailed information on gene flow patterns together with levels and effects of inbreeding on offspring are valuable to understand the biology of a species and to sketch scenarios of its future persistence.
In plants, gene flow occurs in two ways, with pollen dispersal followed by the distribution of seed. Since the latter also holds the colonization function, it can greatly influence levels of inbreeding within populations: if seed gene flow is limited and related individuals establish in spatial proximity, matings among relatives can be further promoted.

Natural regeneration of the subalpine conifer species Swiss stone pine (*Pinus cembra*) largely depends on the seed-caching activities of the European nutcracker (*Nucifraga caryocatactes*). The combination of wind pollination with bird-mediated seed dispersal can hence be expected to greatly shape the spatial distribution of genotypes and the genetic diversity within and among populations. Therefore, the overall aim of this study was to investigate in detail gene flow patterns, together with their effects on spatial genetic structure (SGS) and, thus, inbreeding probabilities in an exemplary *P. cembra* population (Rautialp). Furthermore, potential effects of inbreeding on offspring fitness were analysed, hypothesizing that rates of inbreeding and subsequent cumulative inbreeding depression are higher in small and patchily distributed than in large and continuous *P. cembra* populations.

The application of nuclear and chloroplast microsatellite markers on *P. cembra* enables the discrimination of pollen dispersal from seed movements owing to the purely paternal inheritance of the chloroplast genome. Molecular genetic results from the study population Rautialp show that both pollen and seed dispersal occur across the entire stand of nearly 90 ha. Similarly, gene immigration into the stand by pollen and seed was detected (*Chapter I*). These findings support the assumption of extensive gene flow through far-ranging vectors like wind and birds. Nevertheless, we also found strong spatial autocorrelation of individuals up to several tens of metres within the population (*Chapter*

II). This suggests that seed dispersal through nutcrackers occurs in a kin-structured manner, which leads to the establishment of SGS – especially in areas where tree density is low and seed shadows overlap less than in dense areas of the stand. Pollen flow also seems to contribute to this genetic structure, probably through correlated matings. The spatial clustering of related individuals persists from seedlings to adult trees. Given that pollination distance was found to decrease with increasing proximity between potential mates, probabilities for inbreeding are elevated in high-density areas. I additionally detected a seed and pollen source bias: mature trees in areas with high tree density contributed more to regeneration than trees from the low-density area. Taken together, this study suggests that the population Rautialp is vulnerable to the occurrence of high levels of genetic drift and mating among relatives – despite the combination of wind pollination and seed dispersal through a far-ranging bird.

Similar conditions can be expected for other small populations located in the peripheries of *P. cembra*'s distribution range in Switzerland, which are also characterized by relatively small population size, low tree density and limited connectivity. Accordingly, results from the second part of the study show that embryo viability and germination success was significantly lower in open-pollinated offspring from small and peripheral populations, including the stand Rautialp, as compared to those from large and continuous populations in the core of the species' range in the Swiss Alps (*Chapter III*). Since these observations are generally attributed to inbreeding depression in Pinaceae, my results hence support the assumption that these populations are indeed subjected to elevated rates of mating among relatives, with negative effects on natural regeneration.

Thus, this study indicates the relevance of population size and connectivity, even in a long-lived temperate tree species, for which detrimental effects of fragmentation have recently been questioned. At the same time, the thesis reveals the influence of the birds' seed caching activities on the genetic configuration of stone pine populations and consequences for inbreeding probabilities. The study gives valuable information on seed sampling strategies in view of artificial regeneration, which might be necessary for the maintenance of genetic diversity of the species at least in the peripheral ranges.

To conclude, the parallel analysis of molecular genetic markers with differential inheritance in concert with exhaustive sampling and complementation with experimental studies, yields novel and detailed information on the regeneration of a subalpine conifer species occupying a narrow ecological niche.

Zusammenfassung

Verschiedene Prozesse können die genetische Struktur und Diversität einer Population beeinflussen. Der Genfluss innerhalb und zwischen Populationen spielt dabei eine entscheidende Rolle: ist der Austausch von Genen begrenzt, steigt die Wahrscheinlichkeit für genetische Drift und das Auftreten von Verwandtenpaarungen. Dies wiederum kann den Grad der Homozygotie erhöhen, was nachweislich schädliche Auswirkungen auf die Nachkommenschaft verschiedener Tier- und Pflanzenarten hat. Zukünftige Generationen können so eher dem Verlust genetischer Diversität ausgesetzt sein und damit auf lange Sicht ein verringertes adaptives Potential aufweisen. Genaue Informationen über die Muster des Genflusses sowie über das Ausmass und die Auswirkungen von Inzucht auf die Nachkommenschaft sind somit sehr wertvoll, um die Biologie einer Art zu verstehen und Aussagen über ihren Erhalt machen zu können.

In Pflanzen erfolgt der Genaustausch über zwei Wege: auf die Ausbreitung des Pollens folgt die Ausbreitung der Samen. Letzteres hat zusätzlich die Funktion der Besiedlung, und kann sich demzufolge stark auf das Ausmass an Inzucht auswirken: wenn die Samenausbreitung begrenzt ist und sich verwandte Individuen nahe beieinander ansiedeln, so kann dies vermehrt Paarungen zwischen diesen nach sich ziehen.

Während die Ausbreitung des Pollens der subalpinen Konifere Arve (*Pinus cembra*) durch Wind erfolgt, hängt ihre natürliche Verjüngung zum grössten Teil von den Samenausbreitungsaktivitäten des Europäischen Tannenhähers (*Nucifraga caryocatactes*) ab. Diese Kombination aus Windbestäubung mit der Ausbreitung der Samen durch einen Vogel - beides Vektoren, die grosse Distanzen überwinden können - dürfte einen sehr spezifischen Einfluss auf die räumliche Verteilung der Baum-Genotypen sowie die genetische Diversität innerhalb und zwischen Arvenpopulationen haben.

Das übergeordnete Ziel dieser Studie war demnach, detailliert den Genfluss und dessen Auswirkungen auf die räumliche genetische Struktur (SGS) - und somit auch auf Inzucht - am Beispiel eines Arvenbestandes (Rautialp) zu untersuchen. Darüber hinaus wurden potenzielle Auswirkungen von Verwandtenpaarungen auf die Fitness der Nachkommenschaft untersucht, der Hypothese folgend, dass der Genfluss in kleinen, isolierten Beständen stärker begrenzt ist als in grossen, zusammenhängenden Populationen.

Da das Chloroplasten-Genom in *P. cembra* rein paternal vererbt wird, ermöglicht die gleichzeitige Anwendung molekularer Marker aus dem Zellkern- und Chloroplasten-Genom, die Pollen- von der Samenausbreitung zu unterscheiden. Molekulargenetische Ergebnisse des Untersuchungsgebiets Rautialp zeigen, dass sowohl Pollen als auch Samen über das gesamte, ca. 90 Hektar umfassende Gebiet ausgebreitet wurden. Gleichzeitig wurde auch ein Eintrag von Pollen und Samen in den Bestand festgestellt (*Kapitel I*). Diese Ergebnisse unterstützen die Annahme, dass der Genfluss in Arvenpopulationen durch die weit reichenden Ausbreitungsvektoren "Wind" und "Vogel" extensiv ist. Dennoch wurden über Distanzen von mehreren zehn Metern starke räumliche Autokorrelationen zwischen Individuen in der Population festgestellt (*Kapitel II*). Dies lässt vermuten, dass die Samenausbreitung durch den Tannenhäher in einer gerichteten Weise geschieht, die zu einer räumlichen genetischen Struktur führt – insbesondere in Gebieten des Bestandes, wo die Baumdichte niedrig und damit der Überlappungsbereich der Samenschatten geringer ist als in dicht bestockten Gebieten. Auch die Ausbreitung des Pollens scheint einem nicht-zufälligen Muster zu folgen, was damit ebenfalls zu einer genetischen Strukturierung des Bestandes beiträgt.
Die Daten zeigen ausserdem, dass die räumliche Anhäufung verwandter Individuen von der Jungpflanzen- bis zur Generation der Adultbäume erhalten bleibt. Im Hinblick darauf, dass die Bestäubungsdistanz mit zunehmender Dichte zwischen potenziellen Paarungspartnern sinkt, sind Verwandtenpaarungen in Bereichen hoher Baumdichte wahrscheinlich. Zusätzlich wurde ein Ungleichgewicht von Beiträgen an Pollen und Samen zur Nachkommenschaft festgestellt: Adultbäume in dicht bestockten Bereichen tragen mehr zu Nachkommenschaft bei als Bäume aus dünn besiedelten Bereichen.

Zusammengefasst zeigt diese Studie, dass der Baumbestand Rautialp anfällig ist für das Auftreten von genetischer Drift und Inzucht – trotz der Kombination zweier Ausbreitungsvektoren, die einen Genaustausch über grosse Distanzen ermöglichen.

Ähnliche Bedingungen wie im Bestand Rautialp können in anderen kleinen Populationen erwartet werden, die in der Peripherie des Schweizer Verbreitungsareals der Arve liegen. Diese zeichnen sich ebenfalls durch relativ kleine Bestandsgrösse, geringe Baumdichte und begrenzte Vernetzung aus. Dementsprechend zeigen die Ergebnisse des zweiten Teils dieser Studie, dass die Lebensfähigkeit von Embryonen und der Keimungserfolg von offen bestäubten Nachkommenschaften aus diesen kleinen, peripheren Populationen schlechter waren als die aus grossen, zusammenhängenden Beständen (*Kapitel III*). Da solche Beobachtungen in Pinaceae generell einer

Inzuchtdepression zugeschrieben werden, bestätigen meine Ergebnisse die Erwartung, dass die kleinen, peripheren Arvenpopulationen in der Tat erhöhten Verwandtenpaarungen ausgesetzt sind - was wiederum nachteilige Auswirkungen auf die natürliche Verjüngung hat.

Die Studie verdeutlicht somit die Wichtigkeit der Populationsgrösse und der Vernetzung - auch für langlebige Baumarten der gemässigten Breiten, für die kürzlich die nachteiligen Effekte der Fragmentierung in Frage gestellt wurden. Gleichzeitig wird gezeigt, welchen Einfluss das Samenversteckverhalten der Vögel auf die genetische Zusammensetzung und damit auch auf die Inzuchtwahrscheinlichkeit innerhalb einer kleinen Arvenpopulation hat. Die Arbeit gibt wertvolle Information darüber, wie sich die Sammelstrategie von Saatgut auswirkt, was wiederum im Hinblick auf künstliche Verjüngung im Zusammenhang mit der Erhaltung der genetischen Diversität dieser Art wichtig sein könnte.

Mit meiner umfassenden Beprobung des Untersuchungsgebietes Rautialp, der gleichzeitigen Analyse unterschiedlich vererbter molekulargenetischer Marker und der Ergänzung mit einem experimentellen Ansatz, liefere ich somit neue und detaillierte Erkenntnisse über die Fortpflanzungsbiologie der Arve - einer subalpinen Koniferenart, die eine enge ökologische Nische besetzt und sich durch ihre spezielle Verbindung mit dem Tannenhäher auszeichnet.

General Introduction

Background

Genetic diversity is the basis for a populations´ adaptive potential. Several factors like mutation rates, the population's evolutionary history and the species´ biology and mating system can influence its genetic diversity. Furthermore, the dispersal of genes, i.e. gene flow within and among populations, and the spatial distribution of alleles within a population play a crucial role (e.g. Hamrick *et al.,* 1992; Hamrick and Godt, 1996).
Population fragmentation and small population size can erode genetic diversity, with gene flow being restricted within and among populations. Consequently, small and isolated populations are prone to genetic drift and mating among relatives (Young *et al.,* 1996; Lowe *et al.,* 2005; Aguilar *et al.,* 2008), with possible detrimental consequences on offspring fitness (Keller and Waller, 2002) and, in the long term, reduced adaptive potential of the species or population under consideration. If related individuals occur in spatial aggregation, the occurrence of inbreeding might even be further enhanced.

Conifers are commonly characterized by high genetic diversity (Hamrick and Godt, 1996). Furthermore, pollen production and dispersal through wind is extensive, generally promoting wide-ranging gene flow (Koski, 1970; Latta *et al.,* 1998). The predominantly outcrossed mating system (Krutovskii *et al.,* 1995; Rajora *et al.,* 2002; Politov *et al.,* 2008), and their longevity should also facilitate the persistence of genetic diversity in these species. Nevertheless, increased inbreeding was found in small and disjunct conifer populations (e.g. Rajora *et al.,* 2002; O'Connell *et al.,* 2006; Mimura and Aitken, 2007). Due to their high genetic load, inbreeding typically has strong negative effects on embryo viability, seed germination and performance in subsequent life stages (Sorensen, 1969; Sorensen and Miles, 1982; Savolainen *et al.,* 1992).

Swiss stone pine (*Pinus cembra*) is a wind-pollinated, long-lived and primarily outcrossed conifer species (Zoller, 1991; Politov *et al.,* 2008). Furthermore, seed gene flow almost exclusively depends on seed-caching activities of a far-ranging bird, the European nutcracker (*Nucifraga caryocatactes*; Mattes, 1990). Thus, gene exchange can theoretically be expected to be extensive and, hence, spatial genetic structure to be random within populations. However, kin-structured seed dispersal through birds might lead to an aggregation of related individuals, even if long-distance dispersal occurs (e.g. Furnier *et al.,* 1987; Schupp *et al.,* 2002; García *et al.,* 2009). Furthermore, in small and fragmented

populations, reduced mate availability and/or skewed reproductive success of a few individuals might enhance probabilities for inbreeding and local genetic drift.

In Switzerland, the species´ distribution is characterized by large core populations in the central Alps, while stands are small and fragmented in the northern peripheries of the Alps (Furrer, 1955). Previous molecular genetic work showed lower genetic diversity within and higher genetic differentiation among these peripheral populations as compared to large core stands (Gugerli *et al.,* 2009). While gene flow among these peripheral populations thus seems to be limited, the extent of gene dispersal within *P. cembra* stands remains unclear. Furthermore, only little is known about the genetic consequences for the species resulting from seed dispersal through the nutcracker. Since ongoing global warming is predicted to negatively affect the species´ persistence (Casalegno *et al.,* 2010), the investigation of gene flow, spatial distribution of genes and resulting levels of inbreeding in *P. cembra* will reveal crucial information for the conservation and management of the species.

Main objectives of the study

This study aimed to investigate gene flow patterns and the resulting spatial distribution of genes in a peripheral and small stand of *Pinus cembra*. Hypothesizing that small population size and fragmentation, in combination with directional effects of avian seed dispersal, leads to an increase in probabilities for mating among relatives, we also aimed to elucidate potential detrimental effects of inbreeding on offspring of this subalpine conifer species.

In particular, the objectives of the study were

(i) to characterize pollen and seed gene flow within the small and peripheral *P. cembra* stand Rautialp (*Chapter I*)

(ii) to investigate spatial genetic structure within two life stages (adult and juvenile trees), resulting from two-step gene flow processes, on Rautialp (*Chapter II*)

(iii) to elucidate whether detrimental effects of inbreeding are higher in small, peripheral, compared to large, continuous populations of *P. cembra*, and examine whether such effects correlate with inbreeding characterized using molecular markers (*Chapter III*)

(iv) to develop species-specific nuclear microsatellite markers for *P. cembra* as a prerequisite to describe gene flow and genetic structure, and to infer realized mating patterns (*Chapter IV*).

To address the first two objectives, we geographically mapped and sampled adult and juvenile trees within the small and peripheral *P. cembra* stand Rautialp. Parentage analysis of individuals genotyped at loci from two genomes with different inheritance (microsatellites from the biparentally inherited nuclear and the paternally inherited chloroplast genomes) facilitated the parallel investigation of both wind pollination and seed dispersal processes within the area. Since the tree stand on Rautialp comprises areas with varying tree densities and admixture with Norway spruce (*Picea abies*), we were able to investigate gene flow processes in relation to these ecological factors (*Chapter I*).

Spatial autocorrelation analysis using the data set from Rautialp subsequently elucidated the effects of these gene flow processes on the distribution of genes in two-dimensional space. Thus, we were able to investigate patterns of relatedness within and among clusters of individuals in the area, which gives insights into respective consequences for inbreeding probabilities. The characterization of spatial genetic structure (SGS) was done for two life stages (juveniles and adults), thus reflecting both recent but also historic gene flow processes, and again considering tree density and levels of admixture (*Chapter II*).

To achieve objective (iii), we examined inbreeding levels at the earliest life stages through analysing possible inbreeding depression in open-pollinated seeds. We sampled in two different population types, peripheral and core populations in eastern Switzerland, and both molecular genetic analyses and common gardens experiments were conducted. Thus, we could investigate potential relationships between genetic inbreeding coefficients and offspring fitness at the family level between population types, following the hypothesis that inbreeding levels are higher in small and peripheral populations, where mate availability is lower and gene flow is restricted, as compared to large core populations (*Chapter III*).

Previous to the above studies, species-specific nuclear microsatellite markers were developed, using an enriched library method (Edwards *et al.,* 1996), in collaboration with Dr. F. Sebastiani (Dipartimento di Biotecnologie Agrarie) and Prof. Dr. G.G. Vedramin

(Istituto di Genetica Vegetale) at the Università degli Studi di Firenze. Given the large genome size comprising high levels of transposable elements typical of conifer species, substantial efforts were required to obtain a sufficient number of loci for the subsequent analyses (*Chapter IV*).

Chapter I

Pollen- and seed-mediated gene flow in a peripheral population of the bird-dispersed Swiss stone pine (*Pinus cembra*)

Submitted as:
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Pollen and seed mediated gene flow in a peripheral population of the bird-dispersed Swiss stone pine (*Pinus cembra*). Tree Genetics and Genomes.

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Abstract

Gene flow is the main force counteracting genetic drift within populations. Parentage analysis of juveniles may elucidate patterns of gene flow, but mostly suffers from limited resolution when only biparental molecular markers are available, such as nuclear microsatellites. In the present study, we assessed recent realized gene flow by wind pollination and subsequent avian seed dispersal within a delineated stand of *Pinus cembra*, using both biparentally and paternally inherited microsatellite markers. We mapped and genetically analyzed all adult trees (N=816), varying in density and admixture, across an area of nearly 90 ha and natural regeneration (N=800) along ten linear transects set out across the adult stand. Parentage analysis revealed long-distance dispersal of both pollen and seed across the entire stand and extensive gene flow dynamics within the population. Gene immigration occurred mainly through pollen (30 %) but also via seed (11 %). Trees from high-density sectors were pollinated over shorter distances and contributed more pollen and seed to the regeneration than sparsely occurring trees at the upper tree limit. High sibship frequencies within clusters of juveniles and significant heterozygote deficiencies in both adults and juveniles suggest non-random mating and/or spatial sub-grouping within the population. Thus, despite extensive gene flow, effects of differential tree density in combination with caching behaviour of birds cause a sectorial imbalance in the contribution of parental genes to the offspring. In turn, gene flow reaches up to and beyond the tree limit, which may prove beneficial in view of colonization dynamics as a response to rising temperatures owing to climate change.

Introduction

Natural populations are subjected to forces that tend to produce genetic differentiation and to processes that lead to genetic homogeneity. While mutations, genetic drift and selection can increase the genetic differentiation of populations, gene flow through gametes and individuals counteracts this trend. In plants, genes are dispersed in two steps, first by pollen and then by seed, with the latter having a colonization function. The life history traits related to pollen and seed dispersal and, thus, the extent of gene flow, greatly differ among species. The extensive production of pollen and its dispersal by wind, two factors typical for pines, generally promote wide-ranging gene flow (Koski, 1970; Latta *et al.,* 1998). Besides dispersal distance and the amount of pollen produced, its genetic composition in terms of diversity plays an important role for the exchange of genes. While the overlapping of generations in long-lived plants generally increases the genetic diversity in pollen clouds, low tree density and small population size was shown to lead to a reduction in the number of pollen donors and, thus, reduced pollen availability (Knapp *et al.,* 2001; Sork *et al.,* 2002). Furthermore, the admixed trees of other tree species in a forest may act as physical barriers constraining random pollen flow.

Gene flow can also be restricted due to the specific seed dispersal mechanisms of a species. In many tree species, progeny were found to clump around their source tree (Bacles *et al.,* 2006; Bittencourt and Sebbenn, 2007; Scotti *et al.,* 2008; Nakanishi *et al.,* 2009). By contrast, seed distribution through far-ranging animals like birds might disperse seeds over long distances (Vander Wall and Balda, 1977; Mattes, 1990; Jordano *et al.,* 2007), thus promoting gene flow within stands, but also among patchily distributed populations. Bird dispersal can furthermore facilitate colonization of new habitats, which becomes especially important in the light of range shifts as a consequence of ongoing climate change. However, the selective dispersal of seeds from a few source plants, the deposition of seeds at a few preferred sites or a limitation in dispersal distance might lead to restricted gene flow and spatial clustering of related individuals (Furnier *et al.,* 1987; Jordano and Godoy, 2002; Schupp *et al.,* 2002). Hence, animal-induced seed dispersal can be considered a main determinant of the spatial dynamics and the genetic makeup within and among plant populations.

The life-history of Swiss stone pine (*Pinus cembra*), a subalpine European conifer, is characterized by longevity, wind pollination and the production of large, wingless seeds (Zoller, 1991), which are almost exclusively dispersed by the European nutcracker

(*Nucifraga caryocatactes*) (Mattes, 1990). The birds deposit the seeds in caches as a winter food source, and germinating seeds from unrecovered reserves often result in seedling establishment (Tomback *et al.,* 1993). In the closely related *Pinus albicaulis,* individuals were found to be related within resulting multi-stem clusters (Furnier *et al.,* 1987). Such spatial proximity of relatives might in turn promote inbreeding, especially if effective population size is small. We found significantly higher inbreeding depression in embryos from small peripheral populations compared to large core populations in Switzerland (Salzer & Gugerli, submitted). Yet, gene dispersal processes and patterns of relatedness within tree clusters remain unclear in this species. Considering that modelling predicts current global warming to negatively affect *P. cembra* (Casalegno *et al.,* 2010), knowledge on gene flow and potential inbreeding are fundamental to sketch populations' responses to climate change.

In this work, we combine geographical (position) and environmental (tree density, admixture with *Picea abies*) with molecular genetic data (nuclear and chloroplast microsatellites) of seedlings and adult trees in an isolated, peripheral stand of *P. cembra.* Based on parentage analysis, we describe patterns of realized gene flow through wind pollination and subsequent avian seed dispersal. We hypothesized that gene flow was restricted owing to population isolation and to directional and kin-structured seed dispersal through nutcrackers. Our results highlight how gene flow in *P. cembra* counterbalances forces leading to genetic drift, and give insights into the birds´ caching behaviour with consequences for the genetic structure in subsequent stone pine generations.

Materials and Methods

Study species

Pinus cembra L. is a monoecious closed-cone pine of the five-needle subsection *Cembrae* in the subgenus *Strobus* (Liston *et al.,* 1999). Its natural range expands across the European Alps and the Carpathian mountains (Zoller, 1991; Tutin *et al.,* 1993). It grows in high-elevated mountain areas, restricted to a narrow zone from subalpine altitudes to the timberline, often in association with Norway spruce [*Picea abies* (L.) Karst.] and European larch (*Larix decidua* Mill.) (Rikli, 1909; Zoller, 1991; Tutin *et al.,* 1993). Previous studies on the breeding system of the long-lived, wind-pollinated stone pine found predominant outcrossing (Lewandowski and Burczyk, 2000; Belokon *et al.,* 2005; Politov *et al.,* 2008). Though peripheral and small alpine populations revealed higher rates of early inbreeding depression, genotypes of open-pollinated seed confirmed the effective predominance of outcrossing (Salzer and Gugerli, submitted).

Regeneration of *P. cembra* almost exclusively depends on the seed-caching activities of the European nutcracker (*Nucifraga caryocatactes* L.) (Mattes 1990). The corvid birds typically collect seeds from both single- and multiple-cone loads in a sublingual pouch and distribute the seeds to several tens of thousands of caches within their breeding territory. Outstanding orientation abilities allow the birds to re-locate and exploit over 80 % of caches, as these are located mainly on easily accessible microsites such as exposed ridges, protruding rocks, etc. (cf. Kamil and Jones, 1997). Such locations, in turn, typically offer suitable conditions for germination and seedling establishment. Thus, the avian seed dispersal is considered the driving force in the regeneration of the trees (Mattes, 1990), and this dynamics often results in multi-stem tree clusters (Tomback *et al.,* 1993).

Characterization of study stand

We studied a large plot within a small and well-delineated stand of *P. cembra*. Its confined number of trees facilitated an excessive sampling of potential adult trees, and it is considered representative for other populations in the Northern Swiss Alps regarding soil, altitude and climate (Ott *et al.,* 1997).

The subalpine population on Rautialp (47.4°N, 9.0°E; Näfels, canton of Glarus; 1700–1900m a.s.l.; Fig. 1) is located in an East–West oriented Karst area that has been extensively used in past centuries as pasture land and for wood and seed harvesting until it

was declared a forest reserve (personal communication local forest service) in 2002 (Kanton Glarus; http://www.gl.ch/documents/Liste_Waldreservate.pdf. Accessed March 26, 2011). Today, the area is still used for livestock grazing during summer months. The study plot of about 90 ha comprises *P. cembra* at various densities. Sectors West (W) and Centre (C) are characterized by dense occurrences of stone pine, while the trees at the high-elevation end of the study area in sector East (E) are sparsely distributed (Fig. 2a). Stone pines are strongly admixed with *P. abies* in W, while spruce sparsely occurs in C and is virtually absent in E. Small groups of unsampled *P. cembra* trees occur adjacent to the study plot and in several hundred meters distance.

Mapping and sampling

We geo-referenced all adult trees with a diameter at breast height (DBH) ≥12 cm across the entire study plot (N=816; Table 1), using a total station (TPS 1200, Leica). We measured tree height using the tachymeter and determined DBH. In multi-stem trees, we mapped all stems if they were furcated below 1.2 m. For genetic analyses, we sampled fresh foliage from all mapped individuals.

Within the range of the mapped adult stand, we also mapped (etrex Vista, Garmin) all juveniles (i.e. saplings; N=800; Table 1) of 10–50 cm height on ten parallel transects (20 m x 500 m), crossing the study site from North to South in 100-m distance (Fig. 2b). Each occurrence of single or clustered juveniles was considered a sampling site. In case of juvenile clusters, we mapped all individuals if the cluster included at least one juvenile ≤50 cm. W and C comprised two transects each, while we sampled juveniles on six transects in E to compensate for lower juvenile density. We recorded single or cluster occurrences of juveniles and counted the number of juveniles per cluster. For each mapped juvenile, we collected needles for DNA extraction.

Genetic analyses

We used 50 mg of needle tissue for DNA extraction with the DNeasy 96 Plant Kit (QIAGEN, Hombrechtikon, Switzerland), according to the manufacturer's protocol. Nuclear microsatellite (nSSR) analysis followed the protocol of Salzer *et al.* (2009), excluding locus Pc3 owing to difficulties in amplification. No significant linkage disequilibrium among microsatellite loci was detected (FSTAT 2.9.3; Goudet,1995) in the seven loci included in subsequent analyses.

Observed and expected heterozygosities (H_o and H_e, respectively) per nSSR locus were determined using CERVUS 3.0 (Kalinowski *et al.,* 2007) and averaged across multilocus genotypes. To avoid bias owing to potential Wahlund effects, we analysed offspring and adult samples in each sector separately. We subsequently calculated fixation indices as $F_{IS} = 1 - (H_o/H_e)$ (Wright, 1951). Using the program FSTAT 2.9.3, we tested F_{IS} values for significant deviations from zero using 1000 randomizations, assuming Hardy–Weinberg equilibrium. FSTAT 2.9.3 was also used to estimate allelic richness of loci within juveniles and adults per sector.

We further amplified four paternally inherited mononucleotide chloroplast microsatellite loci (Pt15169, Pt26081, Pt36480, Pt63718; Vendramin *et al.,* 1996), following the multiplex amplification and analysis protocol given elsewhere (Gugerli *et al.,* 2009).

Parent–offspring relationships between adult trees and juveniles were analysed using the maximum likelihood-based method implemented in CERVUS 3.0 (Kalinowski *et al.,* 2007). The most likely parental pairs of juveniles were determined by LOD scores on the basis of allele frequencies of nSSRs from all adult trees. Critical LOD scores and confidence levels for the log-likelihood statistics of parentage assignment were estimated using a simulation approach. To run simulations, we used the following parameters: 10 000 simulated mating events; minimum number of genotyped loci equals four; error rate equals 0.01; all adult trees as candidate parents; proportion of adults sampled 0.6914 (default setting in CERVUS) and 80 % significance threshold.

Parentage assignment in combination with spatial coordinates allowed us to investigate sectorial genetic contributions of adult trees to natural regeneration. Because the chloroplast genome is paternally inherited in *P. cembra* (Rüegg, 2006), we used cpSSR haplotypes to determine the most likely pollen donor within the parental pair. To do so, we compared the cpSSR haplotypes of the parental candidates with that found in the offspring. In cases where the juvenile could only be assigned to one parental candidate, a different cpSSR haplotype identified the parent as the seed tree. If two parental candidates were determined and only one parental candidate shared the cpSSR haplotype with the offspring, it was considered the pollen donor, while the second candidate was determined as the maternal tree. In such cases, we could allocate pollen and seed to the sector of origin and calculate respective dispersal distances, i.e. the distance between pollen donor and seed tree and between the seed tree and assigned offspring. We log-transformed these data to test for differences in pollen and seed dispersal distances

among sectors using analyses of variance (ANOVA) with SPSS (SPSS, 2008), including Tukey post-hoc tests among sectors.

Juveniles within a cluster were considered as half-sibs if one parental candidate was identical (most likely the mother tree) and as full-sibs if both parental candidates were identical among juveniles. If a single offspring showed identical parental candidates it was considered as being selfed.

Results

Spatial distribution and genetic variation

The density of *P. cembra* adult trees decreased from West to East, dropping from almost 30/ha in W to about 3/ha in E (Table 1). Likewise, the density of mapped juveniles per ha was remarkably higher in W and C (>140/ha) than in E (<40/ha).

Among seven nSSR loci, between two (Pc35) and 15 (Pc23) alleles occurred across the entire study plot. Mean allelic richness (A_r) was slightly lower in juveniles than in adult trees in all sectors (Table 1). Both life stages revealed the highest mean A_r value in C (adults: 6.85; juveniles: 6.13). On average, about half of the nuclear genotypes were heterozygous in the adults (H_o=0.510), and H_e averaged 0.536 (Table 1), indicating high levels of genetic diversity. In juveniles, average H_e was similar, yet H_o was slightly lower (mean H_o=0.479, mean H_e=0.537; Table 1). This resulted in an F_{IS} value of 0.109 among all juveniles, contrasting an F_{IS} of 0.049 in all adult trees. F_{IS} values differed significantly from zero at both life stages in all sectors (Table 1).

Among four cpSSR loci, we found between three (Pt2608) and seven (Pt15169) alleles. In total, we encountered 18 cpSSR haplotypes, of which one was dominant (45.7 % of juveniles and 47.8 % of adults) and four were exclusively found in juveniles (Table 2). C comprised the lowest number of haplotypes in adults and the highest in juveniles, while E showed the lowest number in juveniles and most haplotypes in adults. Haplotypes occurring only in adults in E were found in juveniles in W and C.

Gene flow and selfing

Parentage analysis resulted in an exclusion probability of 0.991 for the assignment of offspring to a parental pair. Maximum likelihood analysis allowed us to associate 462 (59 %) *P. cembra* offspring to two most likely parents within the study plot, while 242 (30 %) could be allocated to one parent and 84 (11 %) to no parental candidate. In case of

assignment to one parent only, comparing cpSSR haplotypes between juvenile and parental candidate facilitated to identify the parent to be the maternal tree in 65 % of the cases. Otherwise, offspring and parent shared one of the frequent haplotypes (A, B, C or D), making it impossible to distinguish between seed tree and pollen donor. A considerable proportion of the offspring assigned to two parents originated from seed and/or pollen donors that were located within the same sector where juveniles were found (Table 3). Overall, the largest parental contributions came from W (average 41.4 %).

For one third of all juveniles with both parents assigned, we were able to distinguish the seed tree from the pollen donor on the basis of cpSSR haplotypes (N=152). Paternal contributions to the offspring largely varied among the three sectors (Table 3). Fifty percent of the assigned juveniles in W and C had their pollen donor located in the respective sectors, and only small proportions originated from paternal trees in E. By contrast, almost half of the juveniles found in E were fathered by trees in W. Overall, pollen contributions of trees located in E were lowest (18 %), with respective values above 40 % in W and C. Average pollination distance was similar for seed trees located in W and C (<200 m), while trees were pollinated over significantly longer distances in E (average 315 m, p<0.001; Fig. 3).

Maternal contributions to offspring in the three sectors again showed large variation (Table 3). In W and E, about half of the juveniles originated from seed trees in W, and in decreasing proportions from C and E. Origins of juveniles in C were allocated in almost equal proportions to seed trees from all three sectors (Table 3). Overall, seed contributions were highest from trees in W, followed by C. Stone pines in E mothered only one quarter of all juveniles genotyped. Average seed dispersal distances were similar in W and E (<300 m; p=0.227), but significantly lower in C (average 201.7 m; p=0.004; Fig. 4).

Regarding the numbers of individual trees that were assigned in relation to total numbers of trees sampled per sector, values were similar among sectors (Fig. 5). Nevertheless, a larger proportion of these trees in W and C were assigned to offspring in more than one case. Hence, contributions from trees in these sectors to offspring were proportionally higher than those from trees in E. This applied for both seed trees (Fig. 5a) and pollen donors (Fig. 5b).

We identified 13 % of the 462 assigned juveniles to result from selfing, i.e. these juveniles showed two identical parental candidates (Table 3). Most of the selfed offspring were found in W (Table 3). By contrast, the majority of the self-pollinated trees mothering these juveniles was located in E (24 % of assigned trees in E), followed by 18 % in W and

13 % in C. Among juveniles growing in clusters (*N*=230), up to 27 % were half-sibs (C), with an average of 19 % among sectors. Full-sibs were found in 3 % of juvenile clusters among all sectors Thus, 24 % of all juveniles growing in clumps could be considered as siblings.

Discussion

In our study, we found both extensive pollen and seed dispersal throughout the fully mapped study site, but also gene flow from outside the study area, mainly through pollen. We further detected notable differences in pollination distances, pollen and seed contributions, and selfing rates among the three sectors evaluated. In accordance, sibship frequencies within juvenile clusters were high, and F_{IS} values were positive in both life stages. These findings imply that the forthcoming generation of stone pines in this small, peripheral stand may be prone to genetic drift and non-random mating, despite extensive gene flow by wind- and bird-mediated dispersal.

Pollen gene flow

We observed high levels of pollen flow within the *P. cembra* population studied, with both short (min 1.2 m) and long-distance (max 658.7 m) pollination events. Evidently, these ranges refer to outcrossing distances, i.e. excluding self-pollinations. Pollen flow occurred in both directions, from sectors with high *P. cembra* density to sparsely distributed trees and vice versa. These observations agree with earlier assumptions that wind pollination is extensive within tree populations (Koski, 1970; Latta *et al.,* 1998). Average pollination distances in our study were relatively high (>150 m in W and C, 315 m in E) as compared to averages detected in other wind-pollinated species (e.g., 5.43 m in *P. attenuata*, Burczyk *et al.,* 1996; 68 m in *P. densiflora*, Lian *et al.,* 2001; 83 m in *P. sylvestris*, Robledo-Arnuncio and Gil, 2005). Yet, it has to be taken into account that our study population is relatively small and trees are less dense than in the large and continuous stone pine forests in the Central Alps. As lower population density was suggested to increase wind turbulences, and, thus promote pollen movement (Okubo and Levin, 1989; El-Kassaby and Jaquish, 1996), we anticipate that pollen dispersal within large and continuous *P. cembra* stands might substantially differ from what we observed here. On the other hand, we fully mapped an excessively large area as compared to usual

parentage or paternity studies in temperate trees (but see Kamm *et al.,* 2009), which allowed us to empirically identify realized long-distance pollination events.

Despite wide-ranging pollen movement, less than 20 % of assigned juveniles were sired by pollen donors standing in E. This imbalanced contribution remained prevalent even after correction for the number of potential fathers sampled in each sector. Hence, in relation to total numbers of trees sampled, proportionally more trees in W and C were assigned to sire offspring. Numerous aspects may explain this source bias in pollen flow. First, stochastic factors like weather conditions during the phase of pollen release have to be considered (Houle and Filion, 1993, and references therein), in particular prevailing wind directions with regard to potential pollen recipients during benign weather conditions. The lower tree density in E as compared to W and C can also have a relevant influence, in that pollen donors are fewer and pollen availability is lower (Knapp *et al.,* 2001). Furthermore, individual variation in timing and intensity of male flowering and fecundity, but also female receptivity, should be taken into account (Shen *et al.,* 1981; El-Kassaby *et al.,* 1984; El-Kassaby and Reynolds, 1990), but are difficult to assess over large areas.

Pollination distance was remarkably longer for trees in E. The movement of air and, thus, pollen may be affected by horizontal and vertical canopy structure (Digiovanni and Kevan, 1991). Consequently, a decreasing tree density and no physical barriers, e.g. trees from another species, in-between trees are likely to increase wind turbulences and to prevent impeding pollen movement. In line with results from Larsen & Kjaer (2009), Burczyk *et al*. (1996) and Lian *et al*. (2001), our observations thus support a negative association between tree density (conspecifics and/or canopy of other species) and pollen dispersal distance.

Number and distribution of trees can also influence outcrossing rates (see Aguilar *et al.,* 2008). The stigmas of densely standing trees likely receive more foreign pollen during the short period of flower receptivity than spatially isolated trees. Thus, the relative contribution of a given tree to its own pollen cloud and, hence, the probability for self-fertilization increases with decreasing numbers of neighbouring conspecifics. Accordingly, we found the highest proportion of self-fertilization events in E. These results are in line with observations in another pine species, *P. sylvestris* (Robledo-Arnuncio and Gil, 2005), and in an insect-pollinated Rosaceae tree, *Sorbus domestica* (Kamm *et al.,* submitted). In turn, the relatively high selfing rate in the densely populated W might be explained by admixture with *P. abies*. Spruce trees are likely to act as

physical barriers that limit air movement among trees and promote self-pollination in stone pines. However, it should be considered that factors like unequal male fecundity or asynchronous flowering phenology among individuals can mask the effect of spatial isolation on self-fertilization rate.

Seed gene flow

We found evidence for wide ranging effective seed dispersal (max. 729.7 m), with juveniles in the western sector assigned to maternal trees in the East and vice versa. Hence, our results support observational evidence that the nutcracker is responsible for the largest part of dispersal while other seed vectors like mice or gravity are negligible (Mattes, 1990). The fact that we also found established seedlings beyond the occurrence of adult trees furthermore indicates that bird-mediated seed dispersal can facilitate a colonization of new habitats, including a shift in the altitudinal range.

Among all trees sampled per sector, alike proportions of adult individuals were assigned to mother the juveniles. However, similar to the results on pollen flow, relatively more individuals in W and C were assigned more than once to a juvenile, causing a slight bias in maternal contributions to regeneration among sectors. Several reasons might account for this imbalance. First, numbers of bird breeding pairs, and, thus, caching activities, might be higher in areas with high tree density owing to higher seed availability (Mattes, 1990). Considering that trees in E are growing at the regional upper tree line, more disadvantageous abiotic conditions might lead to a lower reproductive success and/or smaller seed size, which would negatively affect both nutcracker activity and germination success (Salzer & Gugerli, submitted). Additionally, microsite heterogeneity and their interaction with genotype can limit the establishment of offspring from particular trees, especially in habitats where environmental variation occurs on a small scale (Hille Ris Lambers and Clark, 2003).

The percentages of full-sibs within assigned juvenile clusters were low in all sectors, while the frequency of half-sibs averaged 20 %. These values are minimum estimates, as not all juveniles within clusters could be genotyped or assigned, respectively. Our data are in line with results from Furnier *et al*. (1987), who showed high relatedness within multi-stem clusters of avian dispersed *P. albicaulis*. Considering that each seed cache results from a visit by only one bird, our finding suggests that nutcrackers collect seeds from one or only a few potentially related trees per seed caching event, while the number of pollen donors per tree is likely to be higher than one. Multi-stem clusters were also found in the

adult stage, implying that several juveniles within clusters are able to reach the adult stage. Therefore, our results indicate a spatial genetic structure, at least on the small scale, which might lead to increased probabilities for inbreeding.

Gene immigration

We found a pollen immigration rate of 30 % and a seed/pollen immigration rate of 11 %. Comparison of results from previous works on gene immigration shows a clear negative relation between distance among fragments and pollen immigration (see references in Bittencourt and Sebbenn, 2007), following the isolation by distance assumptions (Wright, 1943). Accordingly, Gugerli *et al*. (2009) found higher genetic differentiation among and lower genetic diversity in cpSSR haplotypes of peripheral vs. core stone pine populations. This suggests that gene flow among the peripheral stands is low, probably as a consequence of their geographical fragmentation. Therefore, we assume that at least part of immigrant pollen originated from unsampled trees adjacent to the study plot or small tree occurrences in close proximity rather than from neighbouring stands located at several kilometres distance. This is further supported by the low rates of haplotypes unique to juveniles, despite high proportions of pollen immigration.

Observations of long-distance seed dispersal events through nutcrackers (ca. 15 km; Mattes, 1990) suggest that juveniles might still originate from distant peripheral populations. However, it is likely that the majority of their parents are part of the adjacent tree group, assuming that nutcrackers avoid travelling long distances if seeds are available in proximity. This would also explain the similar gene diversity and allelic richness between the juvenile and adult stage. However, analyses of genetic diversity and differentiation among Swiss stone pine populations will help to better describe the extent of seed gene flow among peripheral populations.

Population genetic parameters

Gene diversity did not change from the parent to the offspring generation, supporting the assumption that the study population is not subjected to strong genetic dynamics such as substantial gene immigration from genetically distant sources. In both life stages and in all sectors, H_o was lower than H_e, which resulted in significantly positive F_{IS} values. This might be attributable to a Wahlund effect, due to an existing genetic structure that may not be congruent with the borderlines of the three sectors. However, F_{IS} values were similar among sectors, hence we assume that heterozygote deficits resulted from a substantial

proportion of matings among individuals that are more related than random, despite extensive pollen flow. The fact that these fixation indices were slightly higher in juveniles than in adult trees supports the hypothesis of inbreeding, in line with the common assumption that inbred individuals are selected against between the juvenile and adult phase (Morgante *et al.,* 1993; Krutovskii *et al.,* 1995; Mitton *et al.,* 1997). Considering that *P. cembra* embryos from Rautialp showed high inbreeding depression at earliest life stages (Salzer & Gugerli, submitted), matings among related individuals likely occur to even higher extents than what can be seen in the juvenile cohort.

Conclusions

Our results show gene immigration and wide ranging gene flow of pollen and seed across the entire study site, including seed dispersal beyond the current range of *P. cembra* in the study area. Yet, areas with high *P. cembra* density contributed more to the offspring than areas sparsely occupied by adult trees, suggesting a source bias of pollen and seed. Low effective population size coupled with low mate availability might lead to genetic drift and increased probabilities of non-random mating in small and isolated *P. cembra* populations, with respective negative fitness consequences in the long term. The likelihood that inbreeding occurs can be further enhanced if related individuals are spatially aggregated, as is suggested by the significantly positive fixation indices and the substantial sibship within clusters of juveniles detected in our study stand. While wind pollination in combination with avian seed dispersal favours effective gene dispersal on the one hand, the bias in pollen and seed source might reduce the adaptive potential in the long term, especially if seed dispersal occurs in a kin-structured manner. Yet, seedlings growing above the uppermost adult trees are promising indicators that stone pine has the potential to expand its altitudinal range beyond the current tree line.

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Tables

TABLE 1 Sample numbers, occurrence of tree clusters and genetic estimates for adult and juvenile *Pinus cembra* trees mapped in three sectors (W, C and E) in the study population Rautialp. Given are numbers of adult trees and juveniles genotyped (*N*), size of the area sampled per sector (area), number of individuals per hectare (N_{ind}/ha), mean allelic richness (A_r), observed heterozygosity (H_o), expected heterozygosity (H_e) and Wright's fixation index (F_{IS}). The genetic parameters refer to the nuclear microsatellite data.

	N	**Area (ha)**	**N_{ind}/ha**	***A_r***	***H_o***	***H_e***	***F_{IS}***
Adults							
W	302	10.5	28.7	6.28	0.524	0.547	0.042*
C	284	10.5	27.0	6.85	0.514	0.538	0.044*
E	230	66.5	3.5	6.43	0.491	0.522	0.059*
total	**816**	**87.5**			**0.510**	**0.536**	**0.049***
Juveniles							
W	282	2	141.0	6.04	0.495	0.555	0.108*
C	287	2	143.5	6.13	0.479	0.535	0.105*
E	231	6	38.5	5.17	0.466	0.515	0.094*
total	**800**	**10**			**0.479**	**0.537**	**0.109***

* significantly different from 0 at p=0.05

TABLE 2 Chloroplast microsatellite haplotype frequencies (*f*) and proportions (*p*) found in adult and juvenile *Pinus cembra* trees in the study population Rautialp. N_H represents the total number of haplotypes per sector. Haplotypes occurring exclusively in juveniles are displayed in italics.

	Adults								**Juveniles**							
	W		**C**		**E**		**total**		**W**		**C**		**E**		**total**	
Haplotype	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***
A	136	0.450	139	0.490	115	0.500	390	0.478	124	0.440	131	0.461	108	0.474	363	0.457
B	64	0.212	32	0.113	28	0.122	124	0.152	57	0.202	46	0.162	34	0.149	137	0.173
C	23	0.076	39	0.137	27	0.117	89	0.109	28	0.099	34	0.120	18	0.079	80	0.101
D	32	0.106	34	0.120	39	0.170	105	0.129	42	0.148	45	0.158	44	0.193	131	0.165
E	22	0.073	22	0.077	9	0.039	53	0.065	15	0.053	16	0.056	15	0.066	46	0.058
F	13	0.043	7	0.025	4	0.017	24	0.029	6	0.021	5	0.018	2	0.009	13	0.016
G	5	0.017	5	0.018	1	0.004	11	0.013	--	--	1	0.003	1	0.004	2	0.003
H	3	0.010	6	0.021	1	0.004	10	0.012	2	0.007	1	0.003	4	0.018	7	0.009
I	3	0.010	--	--	1	0.004	4	0.005	6	0.021	1	0.003	--	--	7	0.009
J	1	0.003	--	--	--	--	1	0.001	--	--	--	--	--	--	--	--

TABLE 2 Continuation

	Adults								**Juveniles**							
	W		**C**		**E**		**total**		**W**		**C**		**E**		**total**	
Haplotype	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***	***f***	***p***
N	--	--	--	--	--	--	--	--	*1*	*0.003*	--	--	--	--	*1*	*0.001*
V	--	--	--	--	2	0.009	2	0.002	--	--	1	0.003	--	--	1	0.001
ZH	--	--	--	--	1	0.004	1	0.001	--	--	--	--	--	--	--	--
ZI	--	--	--	--	1	0.004	1	0.001	1	0.003	--	--	--	--	1	0.001
ZJ	--	--	--	--	1	0.004	1	0.001	--	--	--	--	--	--	--	--
ZK	--	--	--	--	--	--	--	--	--	--	*1*	*0.003*	*2*	*0.009*	*3*	*0.004*
ZL	--	--	--	--	--	--	--	--	--	--	*1*	*0.003*	--	--	*1*	*0.001*
ZN	--	--	--	--	--	--	--	--	--	--	*1*	*0.003*	--	--	*1*	*0.001*
N_H	**10**		**8**		**13**		**14**		**10**		**13**		**9**		**15**	

TABLE 3 Results of parentage analysis comprising 800 juvenile and 816 adult trees of *Pinus cembra* sampled within three sectors (W, C, E) of the study population Rautialp. Given are numbers and percentages of genotyped juveniles that could be assigned to two most likely parental candidates with a confidence interval >80% ($N_{juvenile}$); percentages of parental candidates, i.e. seed tree or pollen donor (% parental candidates) contributing to offspring in each sector; numbers of identified seed and pollen donors (N_{adult}), and percentages contributing to offspring in each sector (% seed trees; % pollen donors); the proportion of offspring originating from selfing (*p* self); juvenile clusters with at least two individuals assigned (juveniles in clusters); the proportion of clusters that comprised half-sibs (*p* half) and full-sibs (*p* full).

					Identified seed and pollen donors							**Juveniles in clusters**		
	$N_{juvenile}$	**% parental candidates**			N_{adult}	**% seed trees**			**% pollen donors**			***p* self**	***N***	***p* half**	***p* full**
		W	**C**	**E**		**W**	**C**	**E**	**W**	**C**	**E**				
Offspring sector W	175 (62 %)	45.7	36.3	18.0	65	50.0	31.5	18.5	50.0	42.6	7.4	0.17	84	0.17	0.00
Offspring sector C	163 (57 %)	37.0	35.8	27.2	67	36.7	30.0	33.3	31.7	50.0	18.3	0.10	83	0.27	0.05
Offspring sector E	124 (54 %)	41.1	26.2	32.7	42	47.4	28.9	23.7	42.1	26.3	31.6	0.09	63	0.13	0.03
Total	**462 (58 %)**	**41.4**	**33.4**	**25.2**	**174**	**44.1**	**30.3**	**25.7**	**40.8**	**41.4**	**17.8**	**0.13**	**230**	**0.19**	**0.03**

Figures

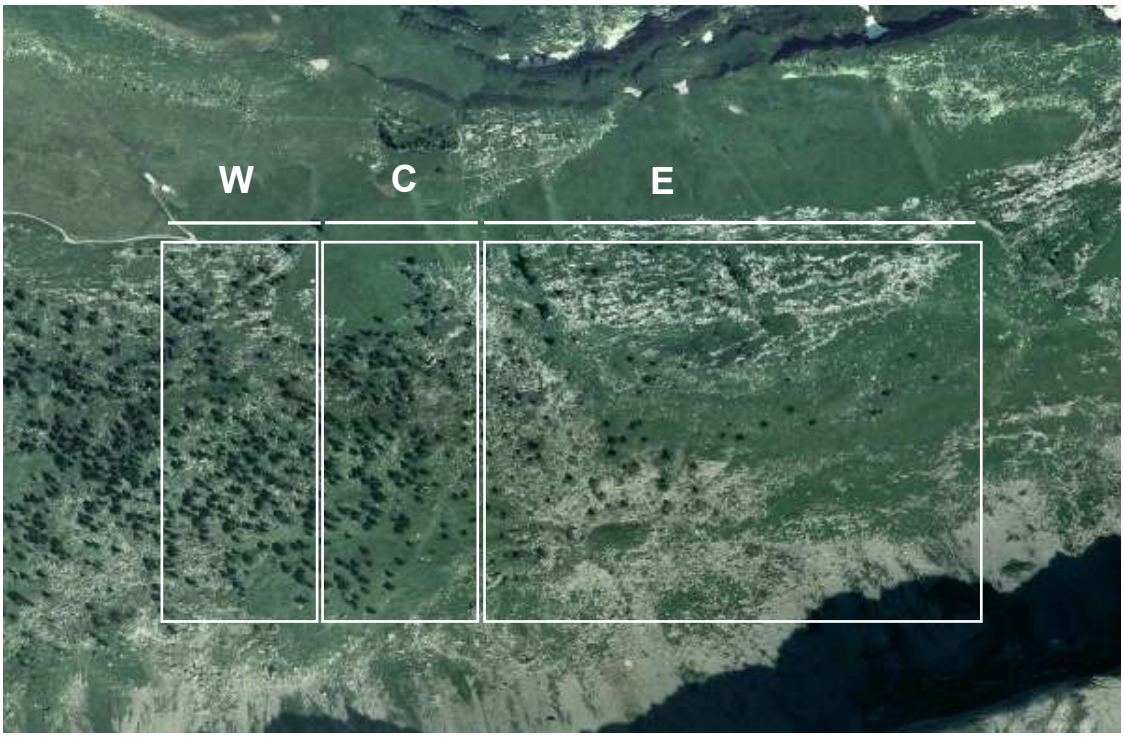

FIG. 1 Orthophoto of the *Pinus cembra* study population Rautialp in the Swiss Alps. White rectangles represent the sampling sectors West (W), Centre (C) and East (E). (Swisstopo Flugdienst/KSL, Dübendorf, 2000).

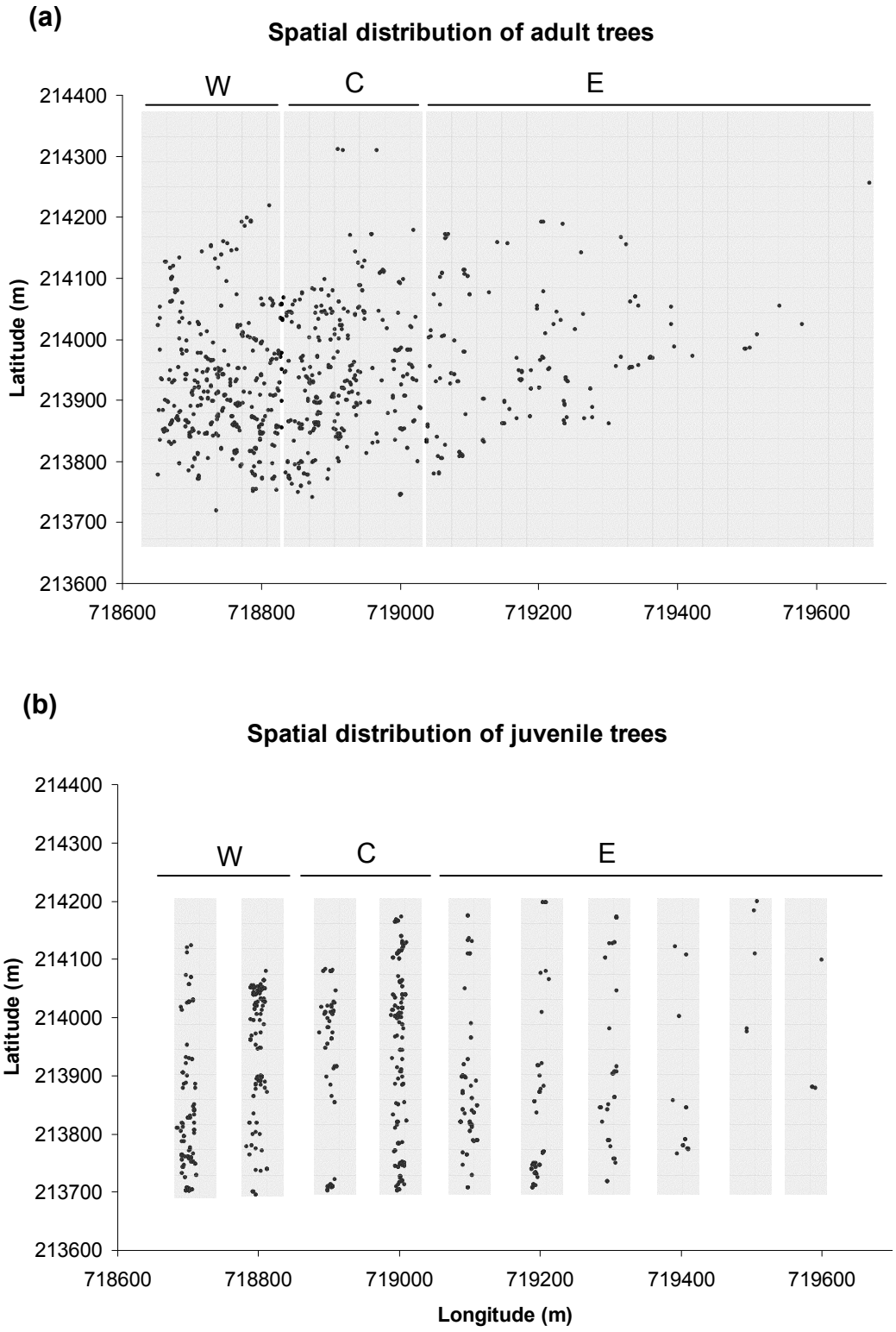

FIG. 2 Spatial distribution of adult (a) and juvenile (b) *Pinus cembra* individuals in the study population Rautialp. Grey areas highlight three sectors (W, C and E) in (a) and ten transects within these sectors where juveniles were sampled in (b). Longitude and latitude refer to the Swiss national co-ordinate system.

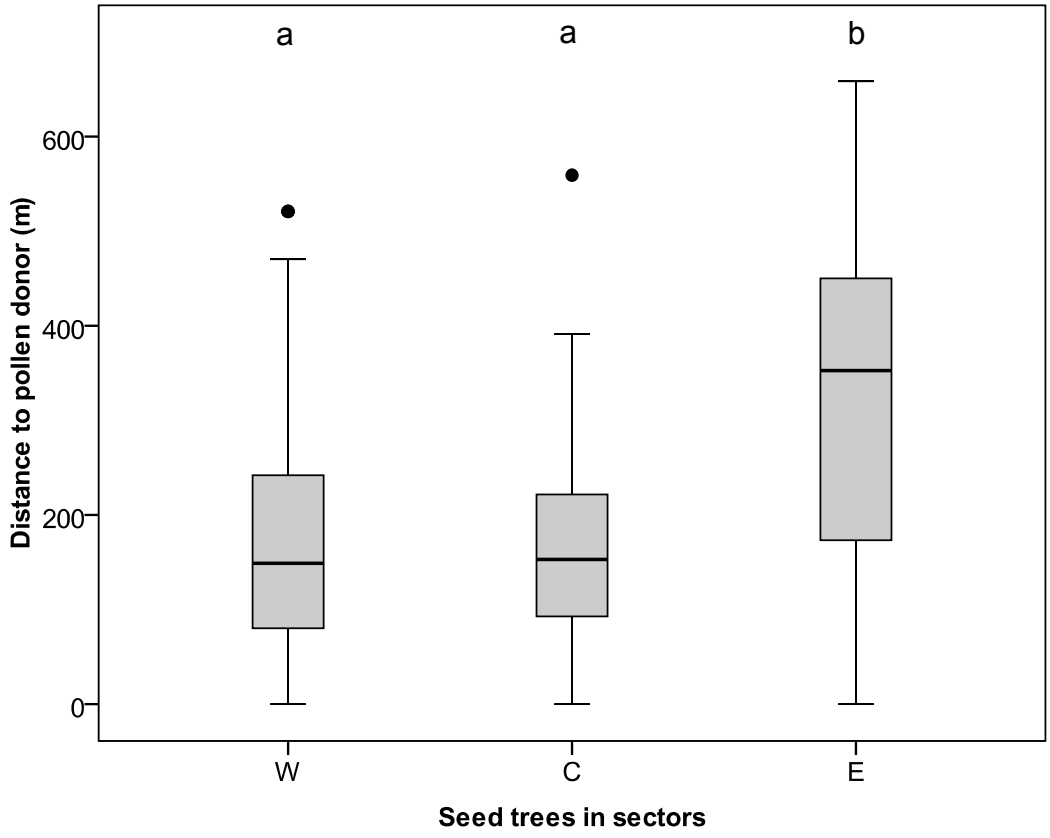

FIG. 3 Boxplots of pollination distances of *Pinus cembra* in Rautialp, i.e. distance between seed trees and pollen donors identified from log likelihood-based parentage analysis of juveniles. Categories refer to the location of seed trees in sectors W, C and E, respectively. Different letters denote between-group significant differences in ANOVA post-hoc tests (p<0.001).

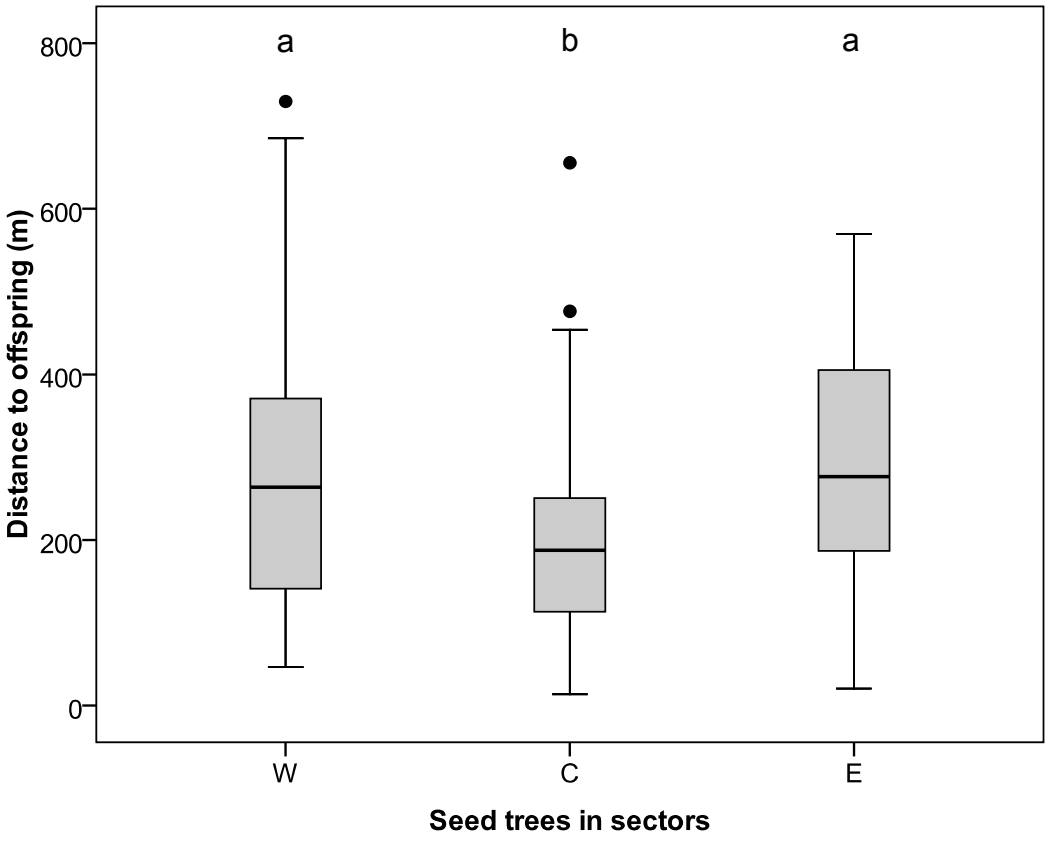

FIG. 4 Boxplots of seed dispersal distances of *Pinus cembra* in Rautialp, i.e. distance between offspring and seed trees identified from log likelihood-based parentage analysis of junviles. Categories refer to the location of seed trees in sectors W, C and E, respectively. Letters denote between-group significant differences in ANOVA post-hoc tests ($p<0.01$).

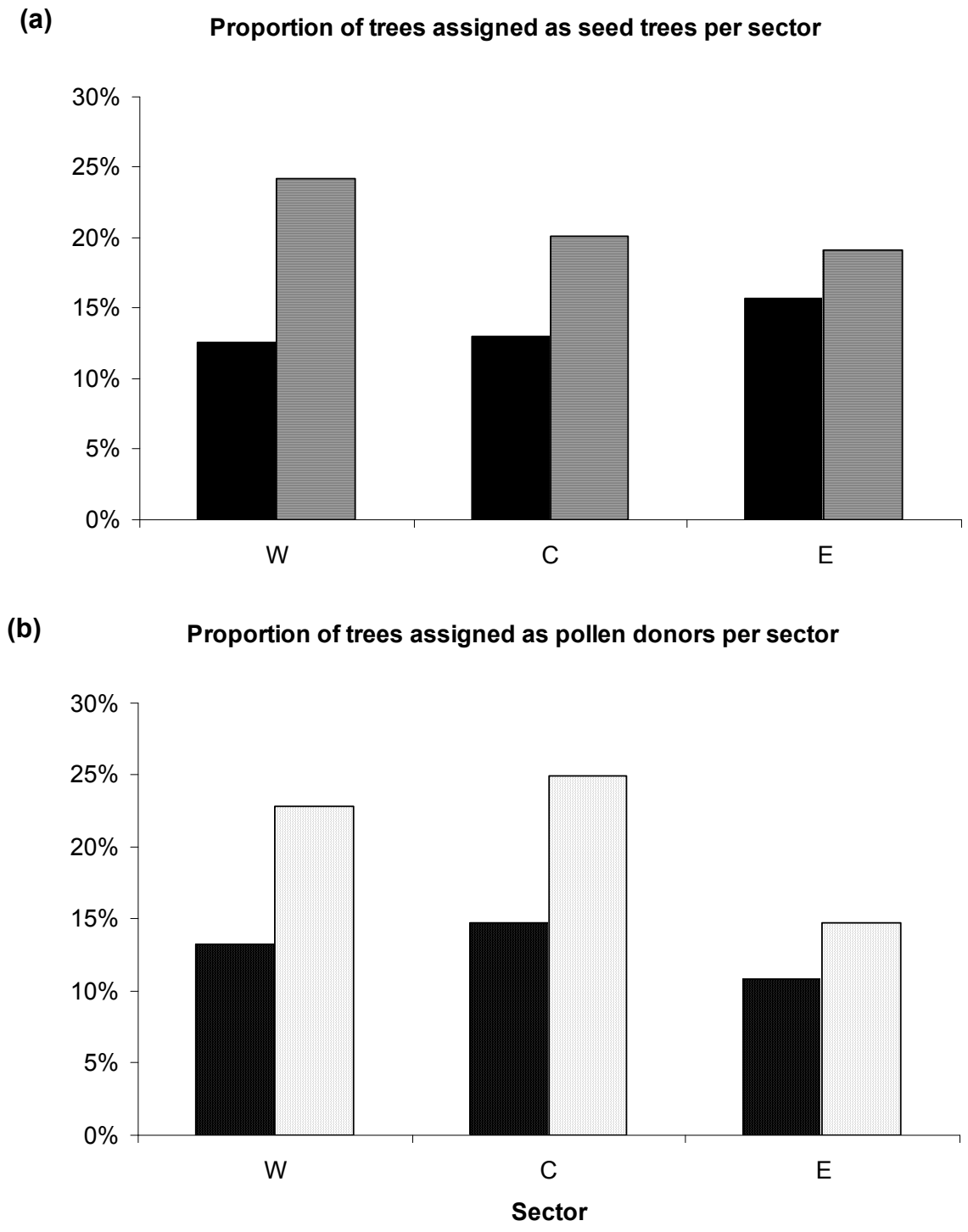

FIG. 5 Proportions of *P. cembra* adult trees assigned to regeneration among total numbers of adult trees sampled per sector in Rautialp, with (a), trees assigned as seed source, and (b), trees assigned as pollen donors. Black bars represent proportions of single individuals that were assigned among total numbers of trees sampled. Grey/white bars consider the proportional frequency, i.e. of how often these individuals were found to mother/father offspring.

Chapter II

Directional effects of bird-mediated seed dispersal in a peripheral population of Swiss stone pine (*Pinus cembra*)

Submitted as:
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Directional effects of bird-mediated seed dispersal in a peripheral population of Swiss stone pine (*Pinus cembra*). Annals of Botany.

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Abstract

Various processes can influence the spatial genetic structure (SGS) within plant populations. Highly important, restricted gene flow might lead to non-random dispersal of genotypes. Hence, increased probabilities for inbreeding may reduce offspring fitness. *Pinus cembra* is a largely outcrossed conifer species, with wind-pollination and bird-mediated seed dispersal facilitating extensive gene exchange. Nevertheless, small peripheral populations showed higher inbreeding depression compared to large stands in the species´ core range. Hence, we studied SGS within a fragmented population to better understand processes possibly affecting mating patterns in *P. cembra*.After mapping juvenile and adult trees in a small, peripheral *P. cembra* population, sampled under varying tree densities, we investigated SGS on biparentally inherited nuclear microsatellite loci, indicative of combined pollen and seed gene flow. The parallel investigation of SGS on paternally inherited chloroplast SSRs facilitated the exclusive investigation of pollen dispersal processes. We found a non-random distribution of genotypes within both life stages. Moran's *I,* indicative of spatial autocorrelation, was highest in short-distance classes comprising multi-stem individuals. SGS in nuclear loci was found up to 85m in adults and 60m in juveniles. Spatial autocorrelation in juveniles occurred with highest frequency in areas with low adult tree density, while this trend was not obvious in adults. The patterns of SGS were similar for chloroplast haplotypes, yet less pronounced.

We infer that kin-structured seed dispersal leads to a clustering of related individuals, especially if adult tree density is low. Despite wind pollination and extensive pollen production, spatial autocorrelation also exists among paternal genotypes. Thus, our study demonstrates that probabilities for matings among related individuals are high in the fragmented study population. Based on our findings, we provide practical recommendations, e.g. for seed sampling strategies dedicated for artificial regeneration.

Introduction

A spatial genetic structure (SGS) is present within a natural population when the genotypes of individuals are non-randomly distributed in two-dimensional space. Following the theory of isolation by distance (Wright, 1943), genetic similarity among neighbouring individuals is in this case higher than among more distant individuals. Such genetic structure can have important ecological and evolutionary consequences for a population at the local scale. It influences population dynamics and persistence by affecting levels of adaptation to micro-environmental variation (Epperson, 1992), and can influence the intensity of density-dependent processes (Hamrick *et al.,* 1993), mating patterns (Young and Merriam, 1994), effective population size and inbreeding and, thus, progeny fitness (Schnabel *et al.,* 1998; Keller and Waller, 2002). All these effects may have severe consequences for the long-term persistence of a population, in particular if it is small and isolated so that genetic drift is high and gene flow limited.

Spatial genetic autocorrelation is the result of interactions among various factors within plant populations: ecological drivers such as mating system (Hamrick and Godt, 1996; Vekemans and Hardy, 2004), density and distribution of adult trees (e.g. Doligez *et al.,* 1998; Gonzales *et al.,* 2010), or habitat variation (e.g. Prentice *et al.,* 2000; García *et al.,* 2009) as well as population fragmentation (Gapare and Aitken, 2005; de Lucas *et al.,* 2009) need to be considered in concert with evolutionary forces like genetic drift and natural selection (Slatkin, 1973; Epperson, 1990). At a fine spatial scale, however, limited gene dispersal is considered a prevalent cause for the formation of local pedigree structures. Thus, only a weak and often non-significant SGS was found in stand-forming tree species with wind-dispersed pollen and seeds (Vekemans and Hardy, 2004). By contrast, fine-scale genetic structure was detected in forest tree species with limited dispersal abilities (Berg and Hamrick, 1995; Dutech *et al.,* 2002), in species exhibiting low adult tree densities (Latouche-Hallé *et al.,* 2003; Oddou-Muratorio *et al.,* 2004; Vekemans and Hardy, 2004) or within fragmented populations (Gapare and Aitken, 2005; de Lucas *et al.,* 2009). A combination of short- and long-distance dispersal in the two vectors of gene flow in plants may thus invoke opposing effects on the shaping of SGS within a population.

Pinus cembra is a long-lived conifer species occurring in subalpine habitats in the European Alps and Carpathian mountains (Rikli, 1909; Tutin *et al.,* 1993). A climate-induced negative population trend was suggested to explain the species' decline in the past

8000 years, and additional extensive anthropogenic exploitation has furthermore led to the occurrence of fragmented and small stands in the northern periphery of the Swiss Alps (Furrer, 1955). The species is wind-pollinated (Zoller, 1991), predominantly outcrossed (Lewandowski and Burczyk, 2000; Politov *et al.,* 2008; Salzer and Gugerli, submitted) and characterized by a distinct life history trait: dispersal of large, wingless seeds and regeneration vastly depend on the caching activities of the European nutcracker (*Nucifraga caryocatactes*) (Mattes, 1990). The bird deposits several thousands of seeds annually as a future food resource, and germination of seeds in caches that are not recovered often results in the establishment of multi-stem tree clusters (Tomback *et al.,* 1993). Individuals within these clusters proved to be highly related (cf. Furnier *et al.,* 1987; Salzer *et al.,* submitted), which likely results in elevated probabilities for inbreeding once these trees are mature. While long-distance seed dispersal through a wide-ranging vector like birds may lead to a decrease in SGS, caching seeds among a few available or preferred sites (García *et al.,* 2009), at a restricted distance (Dick *et al.,* 2008) or selectively dispersing seeds from a few source trees (Furnier *et al.,* 1987; Schupp *et al.,* 2002; Jordano *et al.,* 2007) might promote spatial aggregations of related individuals. Thus, the bird's caching behaviour can be expected to further shape the genetic structure of *P. cembra* populations, especially if effective population size is small and populations are isolated. We therefore extensively sampled and mapped juvenile and adult trees in a peripheral population of *P. cembra* in the Swiss Alps. The parallel analysis of spatial autocorrelation in paternally and biparentally inherited genetic markers facilitates investigating the effects of both wind-mediated pollen and subsequent seed gene flow via bird dispersal. Hence, the aim of this study was to examine how local gene flow affects fine-scale genetic structure and, thus, potential close-relative inbreeding in *P. cembra*. Furthermore, the conditions at the study site allowed us to characterize SGS under varying adult tree densities and levels of admixture with *Picea abies*. Regarding foreseen negative impacts of global warming on the species (Casalegno *et al.,* 2010), such information is relevant for conservation and management of *P. cembra*, especially since peripheral populations revealed higher inbreeding depression compared to large and continuous stands from the core range in the Swiss Alps (Salzer and Gugerli, submitted).

Materials and Methods

Study species

Pinus cembra L. is a monoecious closed-cone pine of the five-needle subsection *Cembrae* (Liston *et al.,* 1999) in the subgenus *Strobus*. It grows in high-elevated mountain areas from subalpine altitudes to the timberline, with its natural range expanding across the European Alps and the Carpathians (Zoller, 1991; Tutin *et al.,* 1993). As described before, *P. cembra* is long-lived and wind-pollinated, and previous studies found a predominantly outcrossing breeding system (Lewandowski and Burczyk, 2000; Belokon *et al.,* 2005; Politov *et al.,* 2008). Nevertheless, higher rates of early inbreeding depression were found in peripheral and small populations as compared to stands in the contiguous core range in the Alps (Salzer and Gugerli, submitted).

Dispersal of *P. cembra* seeds largely depends on the seed-caching activities of the European nutcracker (*Nucifraga caryocatactes* L.). The corvid birds typically distribute pine seeds in several tens of thousands of caches within and outside their breeding territory as a winter food source (Mattes, 1990). These caches are located mainly on easily accessible microsites such as exposed ridges, protruding rocks, etc. (cf. Kamil and Jones, 1997). Such sites protect seeds from secondary predation and typically offer suitable conditions for germination and establishment, while juvniles are also less prone to suffer from fungal infections during early establishment (Senn, 1999). Since approximately 20 % of the caches are not recovered, the avian seed dispersal is considered the driving force in the regeneration of the trees (Mattes, 1990). The establishment of individuals from bird caches often results in multi-stem tree clusters (Tomback *et al.,* 1993) which consist to a large proportion of siblings (Salzer *et al.,* submitted).

Characterization of study species and sampling procedure

This study was conducted in Rautialp (47.4°N, 9.0°E; Näfels, canton of Glarus, 1700-1900m a.s.l.), a Karst area with one of the delineated *P. cembra* stands in the peripheries of the species' distribution in Switzerland. The site is characterized through different densities of *P. cembra* (three to almost 30 adult trees/ha), and trees are increasingly admixed with *Picea abies* in the western, low-elevation part of the stand. The study plot was part of a previous study (Salzer *et al.,* submitted) where it was separated into three sectors. Sectors West (W) and Centre (C) represent the areas with dense *P. cembra* occurrences, with W also comprising substantial numbers of *P. abies* trees, while sector

East (E) is characterized by sparsely distributed stone pines (Fig. 1). We geographically mapped and sampled all adult *P. cembra* trees with a diameter at breast height (at 1.3 m) ≥12 cm in the entire area of nearly 90 ha. In case of multi-stem trees, we mapped all stems if they were furcated below 1.2 m. Furthermore, we mapped and sampled all saplings (i.e. juveniles) between 10 and 50 cm height within ten transects crossing the study site from North to South in 100 m distance (20 m width x 500 m length; Fig. 1). In case of juvenile clusters, we mapped all individuals if the cluster included at least one juvenile ≤50 cm.

Laboratory analyses

Needles sampled from adult trees (N=816) and saplings (N=800) were used for DNA extractions with the DNeasy 96 Plant Kit (QIAGEN), following the manufacturer's instructions. We genotyped all samples at seven nuclear microsatellite (nSSR) loci (Salzer *et al.,* 2009, excluding Pc3) and at four paternally inherited chloroplast (cp) SSR loci (Pt15169, Pt26081, Pt36480, Pt63718; Vendramin *et al.,* 1996), following the protocol given elsewhere (Gugerli *et al.,* 2009).

Statistical analyses

We investigated the spatial genetic structure within adult trees and within saplings in the three sectors with spatial autocorrelation analysis, using Moran's *I*. The analyses were conducted on the basis of both nSSRs and cpSSRs data with the program SPAGeDi 1.3 (Hardy and Vekemans, 2002). Following the authors' guidelines, we defined 21 and 18 continuous distance classes in adult trees and juveniles, respectively. To account for individuals' different spatial distributions, distance classes varied between the two life stages. Spatial autocorrelation analysis was conducted separately for data sets of all three sectors. Moran's *I* values were averaged over loci within each spatial distance class and plotted against the geographical distance for visualization of patterns of spatial genetic structure. By running 10 000 permutations, we determined confidence intervals of 95% under the assumption of random spatial distribution of genotypes. Using a two-tailed significance test, we determined whether the observed value differed significantly from the expected value under the null hypothesis of no spatial genetic structure.

Results

Moran's *I* values averaged among nSSR loci revealed highly significant relationships ($P<0.01$) both among adult trees and among juveniles within the first distance classes in all three sectors (Fig. 2). The within-population genetic structure in the following distance classes slightly differed within and among life stages. In juveniles sampled in W and C, values declined strongly and showed few significantly positive values after the first distance class. In contrast, high and significantly positive Moran's *I* values were found up to a distance of 60 m within juveniles in E. In larger distance classes (>60 m), values were inconsistent but close to zero in all sectors. Nevertheless, some values were significantly negative and alternated with occasional significantly positive relationships within sectors.

Similar to the pattern within juveniles, Moran's indices were highest within adults grouped into the first distance class. However, absolute values were lower than those estimated in juveniles. Significantly positive relationships were found up to a distance of 85 m among pairs of individuals (E), and values also decreased in larger distance classes (>100 m), with several significantly negative values. Unlike in juvenile trees, the frequency of significant values was highest in the west (W) of the ISP. Furthermore, indices within adults did not show the fluctuation from positive to negative values found within juveniles, yet continuously decreased with increasing distance.

The SGS pattern detected in the cpSSR data was similar but less pronounced than the one in nSSR loci, both in juvenile and adult trees. In both life stages, Moran's *I* values were highest in the first distance class and indices decreased in larger distance classes. Similar to the pattern found within juveniles at nSSR loci, a fluctuation from negative to positive values was evident in both life stages.

Discussion

In this study, we conducted spatial autocorrelation analysis within juvenile and adult *P. cembra* trees, genotyped at nuclear and chloroplast microsatellite markers in a small population situated in the peripheries of the species' range in the Swiss Alps. We examined how wind-pollination in combination with bird-mediated seed dispersal may affect fine-scale genetic structure in two contrasting life stages and, hence, consanguineous mating in *P. cembra*. Our results show fine-scale clustering of related individuals in both life stages and both marker types. Thus, gene dispersal through far-ranging vectors like wind and birds does not completely prevent the occurrence of spatial aggregation of relatives in a small and peripheral pine population. This non-random spatial distribution of genotypes can be expected to affect levels of inbreeding within the population.

SGS despite extensive gene dispersal

Unlike studies on other conifer species (Epperson and Allard, 1989; Xie and Knowles, 1991), our results show a spatial autocorrelation of nSSR genotypes within up to several tens of metres in both life stages, and a similar yet less pronounced pattern detected at cpSSR loci. Such SGS has been described in wind- and gravity dispersed species (Berg and Hamrick, 1995; Bittencourt and Sebbenn, 2007; Scotti *et al.,* 2008; Nakanishi *et al.,* 2009) and has predominantly been explained with restricted gene dispersal.

P. cembra, however, is characterized by a combination of wind-pollination and subsequent seed dispersal by the nutcracker. Thus, the distribution of offspring could be expected to be random, since pollen production and dispersal is considered to be extensive in wind-pollinated pines (Latta and Mitton, 1997, 1999; Richardson *et al.,* 2002), and seed transport across distances of up to 15km has been observed in *N. caryocatactes* (Mattes, 1990). In line with this, paternity analysis on regeneration in Rautialp showed extensive pollen and seed dispersal across the entire study site (Salzer *et al.,* submitted). Nevertheless, the present results clearly show a non-random distribution of genotypes, with the most pronounced spatial autocorrelation in the shortest distance class. Here, Moran's *I* values were highly significant at biparentally inherited nSSRs in both life stages, with very high values estimated among juveniles. Since the first distance class comprises individuals in multi-stem clusters, our results imply that seeds within one cache have been gathered from one or only a few trees by the birds (Furnier *et al.,* 1987).

Resulting sib ship among juveniles hence likely explain high Moran's *I* values within clusters. At the same time, one can hypothesize that birds collected these seeds from related source trees in spatial proximity, since SGS was also found among adult trees.

Regarding paternal contributions to the offspring's nuclear and chloroplast genome, it has to be taken into account that genes from pollen donors are initially dispersed via pollen, prior to being moved through the seed. Pollen dispersal largely depends on wind turbulences influenced by tree density and landscape structure (Okubo and Levin, 1989; El-Kassaby and Jaquish, 1996). In line with this, pollination distance decreased with increasing tree density in Rautialp (Salzer *et al.,* submitted). Therefore, offspring may also comprise related paternal contributions in their genomes if seeds were collected from a few mother trees in proximity. The high Moran´s *I* values found at nSSRs and the significant spatial autocorrelation at strictly paternally inherited cpSSR loci in the first distance class strongly support this assumption. In line with this, parentage analysis furthermore proved high proportions of sibships within clusters (Salzer *et al.,* submitted). Thus, pollination among trees in proximity, followed by the kin-structured seed dispersal by birds, may contribute to SGS through correlated matings, especially in areas with high adult tree density. Accordingly, highest Moran's *I* values at cpSSRs were found within W, which is characterized by high density and admixture with *P. abies*. Significant spatial autocorrelation was also found in the first distance class in E, though, the sector with low adult tree density. Nevertheless, parentage analysis revealed extensive seed transport from high-density areas into this sector (Salzer *et al.,* submitted). Hence, the occurrence of offspring sired by related trees from W likely explain this spatial clustering of paternal alleles.

Unlike in *P. albicaulis* (Furnier *et al.,* 1987), SGS was also found among clusters of adult and juvenile trees of *P. cembra* at nSSR loci. Therefore, our results suggest that *N. caryocatactes* caches seeds of related individuals within a spatially restricted area. Our assumptions seem consistent with optimal foraging theory, which predicts that animals should forage to maximize energy gain at the lowest energy cost (Schoener, 1971). SGS at cpSSR loci, though, was much less pronounced than that in nSSRs. This suggests that the two-step gene dispersal, i.e. wind pollination with subsequent seed dispersal, weakens the aggregation of paternally related individuals in longer distance classes. However, the fluctuating pattern of Moran's *I* values around zero found at cpSSRs in both life stages, regardless of significance, suggests the regular occurrence of paternal family clusters. Thus, we assume that the number of investigated loci and the dominance of one particular

cpSSR haplotype may have prevented us from better resolving spatial autocorrelation in the study population.

In contrast to results on *Pinus pinaster* (González-Martínez *et al.,* 2002), SGS was still very pronounced within adult trees of *P. cembra*. Absolute Moran's *I* values detected in the first distance classes were lower among adults than among juveniles, suggesting that thinning of related, maybe selfed/inbred individuals, occurs before maturation (see Salzer and Gugerli, submitted, and references therein), which would reduce the relatedness within clusters. Nevertheless, family structure was still prevalent in the following distance classes and, unlike in juveniles, also evident in areas with high tree density. Since generation overlap is expected to substantiate SGS in tree populations (Doligez *et al.,* 1998), this pattern could be explained with higher numbers of age classes comprised within adult trees compared to juveniles. Additionally, other factors like selection processes, microenvironmental variability (Hille Ris Lambers and Clark, 2003; Troupin *et al.,* 2006), and biotic factors like grazing may influence the establishment of SGS during maturation, and hence change the spatial distribution of genotypes. Yet, these processes are difficult to examine experimentally, since long-term investigations and alternative molecular markers are required to elucidate their impacts.

SGS and tree density

The extent of SGS at nSSRs in juveniles strongly increased from West to East, i.e. with decreasing adult tree density. A skewed distribution in reproductive success of trees can enhance spatial genetic correlations by localized patterns of seed dispersal (Schnabel *et al.,* 1998; González-Martínez *et al.,* 2006). Accordingly, a non-random distribution of genotypes could be attributed to a skewed gene contribution to juveniles in E by only a few dominant reproductive individuals, i.e. local genetic drift. This would likely be reflected in lower genetic diversity estimates. Indeed, expected heterozygosity and allelic richness were slightly lower within E (Salzer *et al.,* submitted). Further supporting this assumption, the majority of juveniles sampled in E were found to originate from trees located in the high-density W (Salzer *et al.,* submitted).

The interpretation of SGS patterns within an animal-dispersed species requires to consider numerous underlying factors, such as frugivore caching behaviour, bird abundance and the relative positions of fruiting trees and deposition sites (Jordano *et al.,* 2007). Bird abundance was observed to be higher in areas with high *P. cembra* density (Salzer, K., personal observation; Mattes, 1990). Therefore, lower SGS in high-density

areas might result from higher caching activities, which in turn lead to an overlap of seed shadows (Furnier *et al.,* 1987). In contrast, in case of lower bird abundance and, thus, lower caching activity in low-density areas (E), this randomizing effect would be smaller. Thus, we assume that high adult tree density can counterbalance the structuring effect of directed seed dispersal (Sagnard *et al.,* 2010) not only in wind- or gravity-dispersed (Gapare and Aitken, 2005; Scotti *et al.,* 2008; Nakanishi *et al.,* 2009), but also in bird-dispersed species (Streiff *et al.,* 1998; Jensen *et al.,* 2003; Kelleher *et al.,* 2004; Dutech *et al.,* 2005).

Conclusions

Seed dispersers can dramatically affect both the demographic and genetic make-up of animal-dispersed plant species. In this study, we found non-random spatial distribution of genotypes within juvenile and adult trees within a population of the bird dispersed *P. cembra*. Such relatedness of trees occurring in spatial proximity could have strong impacts on the realized mating system in *P. cembra*, resulting in significant inbreeding due to matings among related individuals standing in spatial proximity. Considering that significantly higher inbreeding depression was found in small and peripheral *P. cembra* populations, among them the study stand Rautialp (Salzer *et al.,* submitted), we conclude that these populations seem vulnerable for inbreeding and local genetic drift, despite wind pollination and seed dispersal by a far-ranging bird.

Our results also provide useful information for future seed sampling strategies, e.g. for artificial regeneration, in peripheral populations. Because fine-scale structure was shown to persist from the seedling to the mature stage, a minimum distance between sampled trees of 100 m is recommended to reduce the likelihood of sampling related mother trees. Seed collections from different plots are suggested to obtain different families. Furthermore, only one individual per multi-stem cluster should be sampled, since individuals are highly related within these clusters and correlated paternity may be high among such seed lots.

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Figures

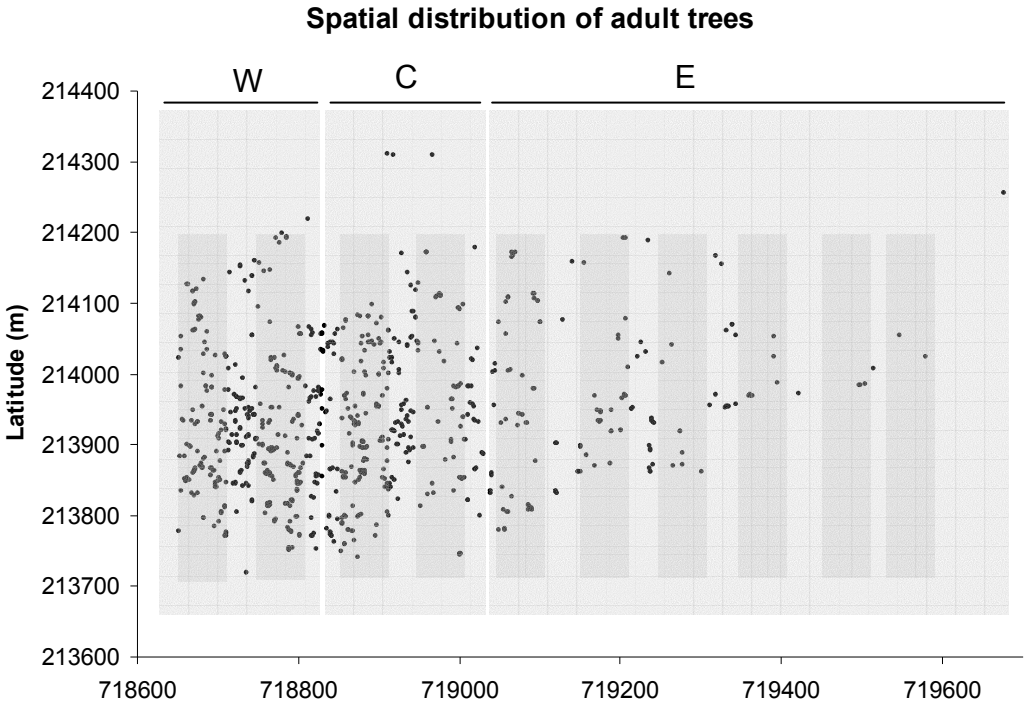
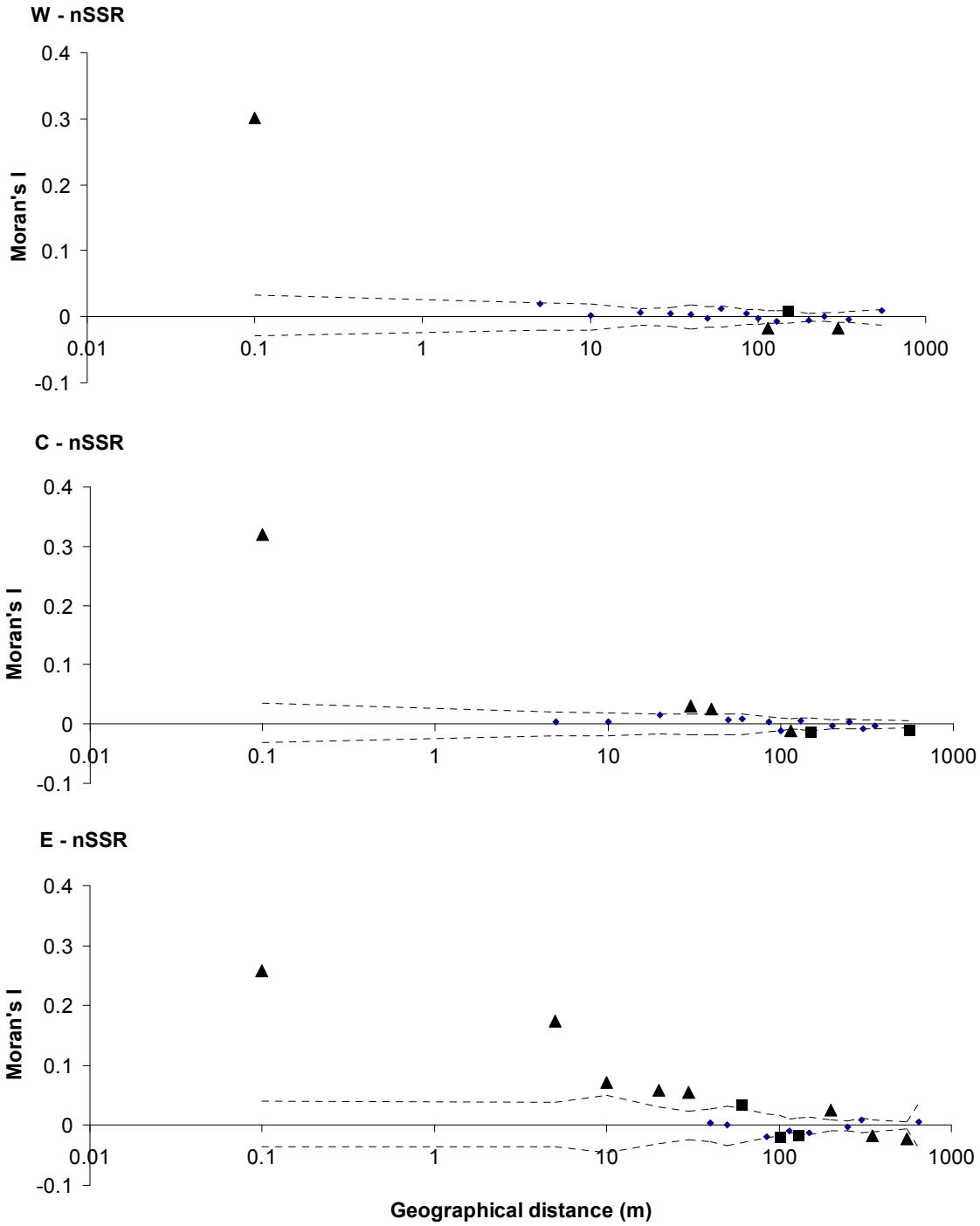
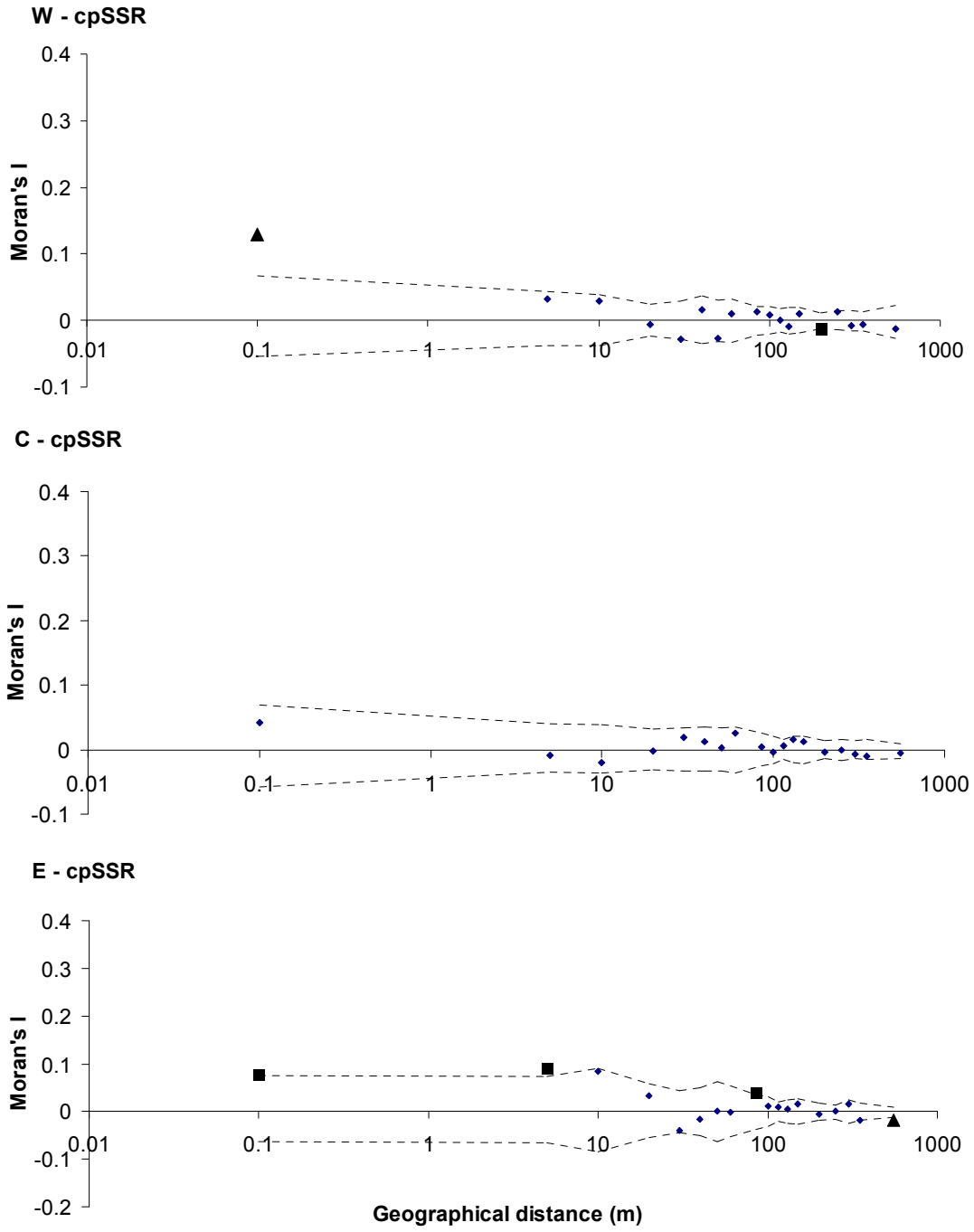


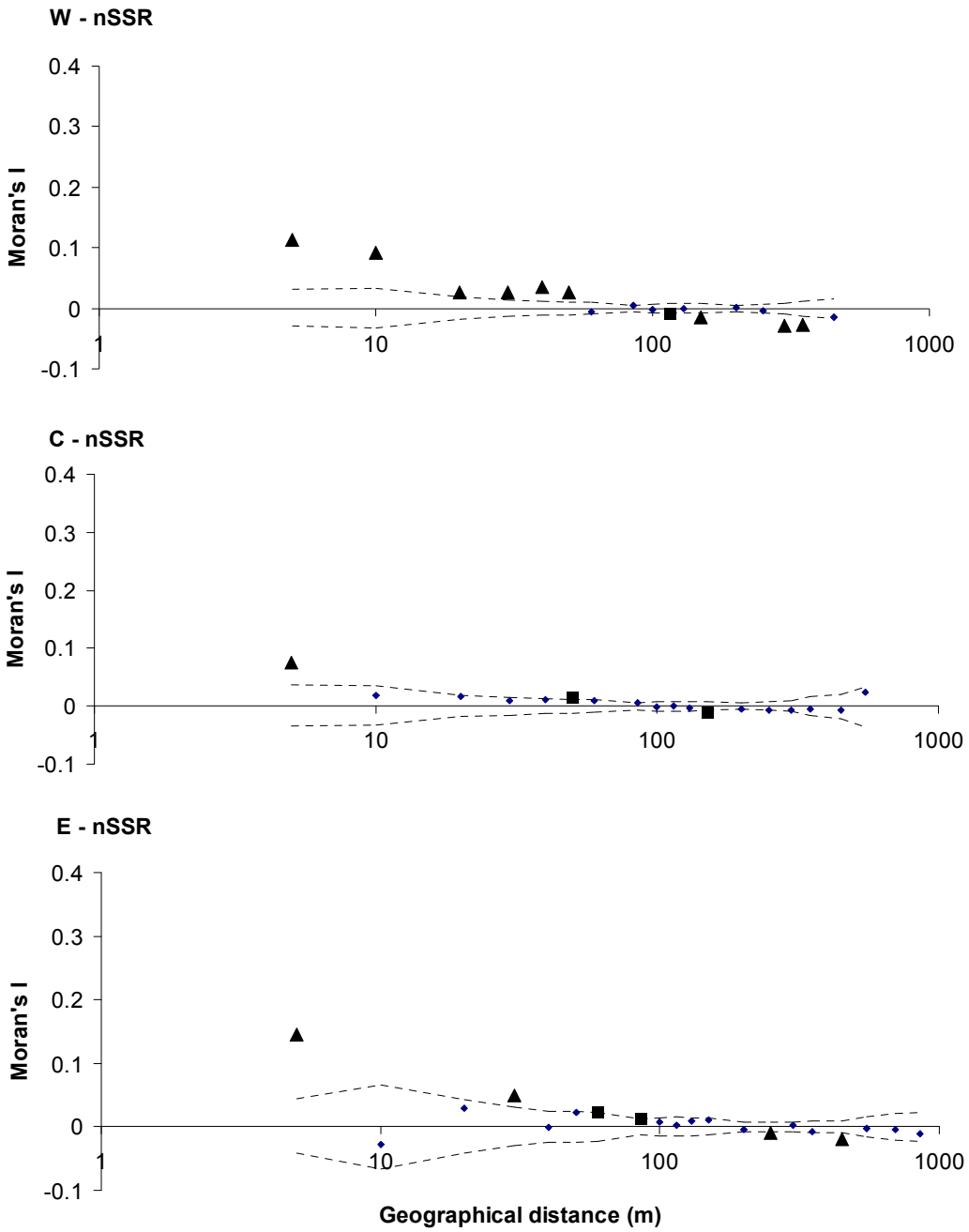
FIG. 1 Spatial distribution of adult *Pinus cembra* individuals sampled within three sectors (W, C and E; highlighted with light grey areas) in the study population Rautialp. Dark grey bars represent ten transects within these sectors where juveniles were sampled. Longitude and latitude refer to the Swiss national co-ordinate system.

(a) Juveniles


W - nSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
0.01
0.1
1
10
100
1000
C - nSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
0.01
0.1
1
10
100
1000
E - nSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
0.01
0.1
1
10
100
1000
Geographical distance (m)


W - cpSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
0.01
0.1
1
10
100
1000
C - cpSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
0.01
0.1
1
10
100
1000
E - cpSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
-0.2
0.01
0.1
1
10
100
1000
Geographical distance (m)

(b) Adults


W - nSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
1
10
100
1000
C - nSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
1
10
100
1000
E - nSSR
Moran's I
0.4
0.3
0.2
0.1
0
-0.1
1
10
100
1000
Geographical distance (m)

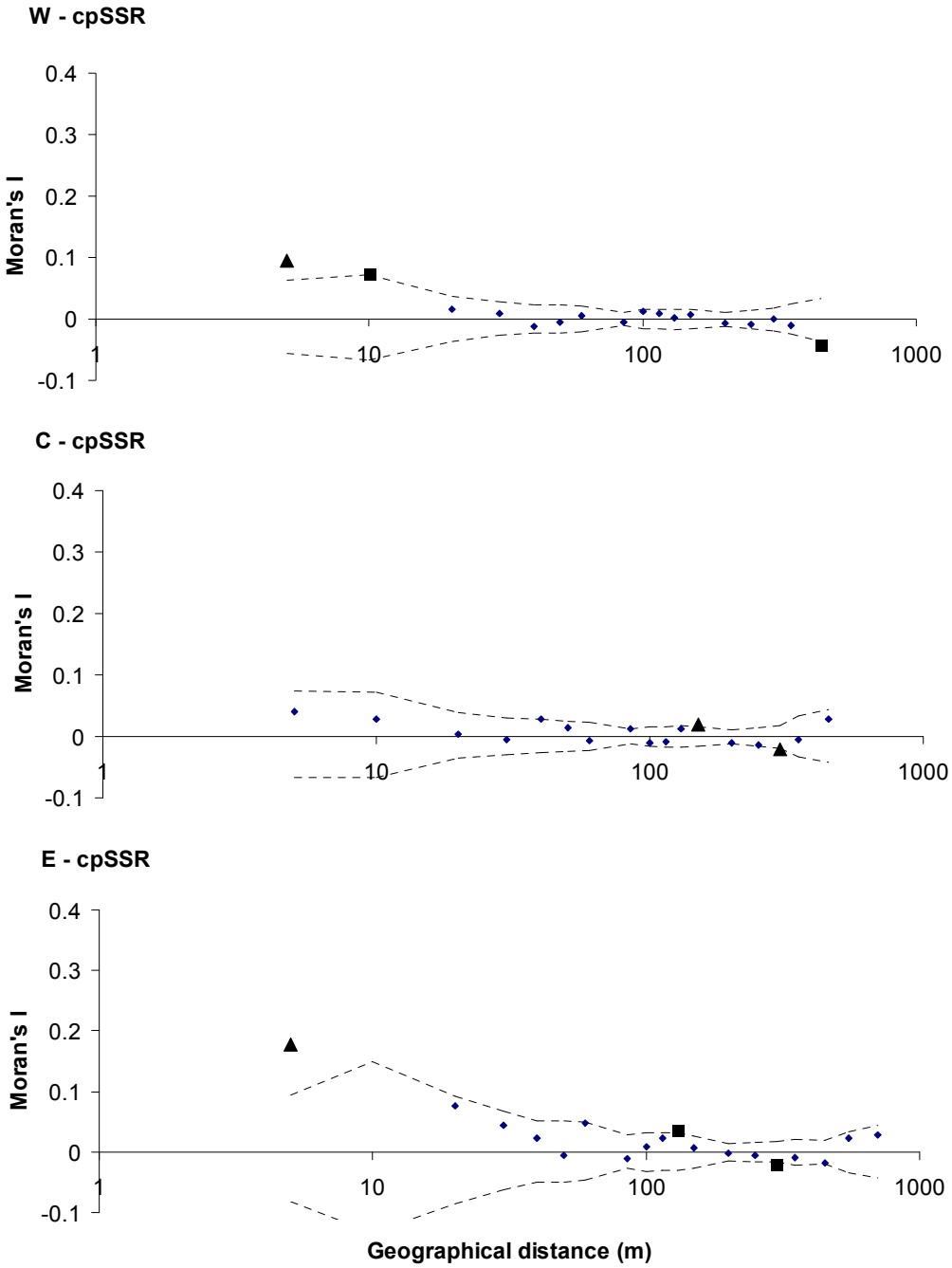


FIG. 2 Spatial correlograms of Moran's *I* for (a) juvenile and (b) adult *Pinus cembra* trees, sampled in three sectors (W, C, E) in Rautialp. Dashed lines represent upper and lower 95 % confidence intervals for Moran's *I* under the null hypothesis that genotypes (nSSR) and haplotypes (cpSSR), respectively, are randomly distributed. Triangles represent a significance level of $\alpha = 1$ %, squares show $\alpha = 5$ %. Note that the x-axis is displayed in a log-scale.

Chapter III

Early-acting inbreeding depression in peripheral populations of Swiss stone pine (*Pinus cembra*)

Submitted as:
Kristina Salzer [1,2] and Felix Gugerli[1].
Early-acting inbreeding depression in peripheral populations of Swiss stone pine (*Pinus cembra*). Annals of Botany.

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Abstract

Small and fragmented populations are prone to mating among related individuals, increasing homozygosity and likely negatively affecting offspring fitness. Swiss stone pine (*Pinus cembra*) forms large and continuous populations in the central Swiss Alps, while stands are small and fragmented in the northern peripheries of the Alps. Global warming is foreseen to negatively affect this species, making information on possible inbreeding depression crucial. Here, we study differences in inbreeding levels and in offspring fitness between core and peripheral populations. We collected open pollinated progenies of *P. cembra* in large, contiguous and in small, isolated populations (core vs. periphery). Part of these seed lots was genotyped at seven nuclear microsatellites to calculate fixation indices. Genetic parameters were then correlated to fitness data obtained in germination experiments conducted with the second half of the progenies. We found significantly lower seed production, higher embryo abortion rates and lower germination success in small peripheral compared to large core populations of Swiss stone pine. By contrast, winter survival and first-year growth of seedlings did not significantly differ between the two population types. Moreover, we found no correlation between any fitness parameter and progeny-derived inbreeding coefficients.

We take these results as an indication of higher inbreeding depression at the earliest life stages in small and fragmented populations, as compared to populations from the contiguous range of Swiss stone pine. Since inbreeding depression mostly affects embryo abortion rates in Pinaceae, molecular markers applied to embryos proofed unsuitable to detect this effect. We infer that population fragmentation negatively affects sexual reproduction in this long-lived, wind-pollinated conifer species occupying a narrow ecological niche. The negative effects of inbreeding on offspring performance and potentially on rejuvenation ask for special attention for the long-term preservation of such species under global change.

Introduction

Small population size and spatial isolation can promote consanguineous mating in natural populations, promoting excess homozygote frequencies in subsequent generations (Allendorf and Luikart 2007; Mimura and Aitken, 2007). Likewise, spatial aggregation of related individuals may lead to increased levels of homozygosity in offspring and the possibility that recessive deleterious or lethal alleles are expressed (Charlesworth and Charlesworth, 1987; Keller and Waller, 2002). Consequently, inbred progeny may show inbreeding depression, i.e. reduced fitness compared to outbred offspring. Inbreeding in predominantly outcrossing conifers has been shown to have strong impacts on the fitness of progeny (e.g. Sorensen, 1969; Sorensen and Miles, 1982), particularly at early life stages, due to the high genetic load typical for conifers (Namkoong and Bishir, 1987; Savolainen *et al.,* 1992). In Pinaceae, inbreeding depression generally results in higher embryo abortion, lower germination success, reduced growth, survival and fecundity when compared to non-inbred individuals (Lande and Schemske, 1985; Kärkkäinen and Savolainen, 1993; Husband and Schemske, 1996; Kärkkäinen *et al.,* 1999). In self-compatible plants such as conifers, homozygosity can also arise through self-fertilization. Accordingly, self-fertilized progeny of Scots pine (*Pinus sylvestris* L.), showed significant inbreeding depression in seed maturation and post-germination survival (Kärkkäinen *et al.,* 1999; Koelewijn *et al.,* 1999). In addition, a study on reproductive fitness traits and mating system in eastern white pine (*Pinus strobus* L.) revealed significantly lower reproductive fitness in small and marginal stands compared to large and central populations (Rajora *et al.,* 2002).

Together with other factors like genetic drift and reduced gene flow, inbreeding in small populations may hamper population persistence in the long term (Ellstrand and Elam, 1993; Keller and Waller, 2002). At the same time, inbreeding may mitigate its negative effects due to selection against inbred offspring, thus, purging deleterious alleles (Lande and Schemske, 1985; Barrett and Charlesworth, 1991; Husband and Schemske, 1996; Sorensen, 2001). In *P. sylvestris*, for instance, populations in northern Finland revealed lower inbreeding depression than populations in the South. Hedrick *et al.* (1999) proposed that these differences might be explained with stronger selection against inbred individuals in northern compared to southern stands. In lodgepole pine (*Pinus contorta* Dougl. ex. Loud.), higher selfing rates partially reduced the negative effects of inbreeding on offspring (Sorensen 2001). However, other findings suggested that purging may

depress population viability through the fixation of deleterious alleles (e.g. Leberg and Firmin, 2008 and references therein).

Swiss stone pine (*Pinus cembra* L.) is a European conifer species of particular interest, as it grows in high-elevated mountain areas, at subalpine altitudes at or close to the timberline, often in association with Norway spruce (*Picea abies* (L.) Karst.) and European larch (*Larix decidua* Mill.; Rikli, 1909; Zoller, 1991; Tutin *et al.,* 1993). Global warming is likely to affect this species, making it more susceptible to interspecific competition (Casalegno *et al.,* 2010). In fact, a climate-driven negative population trend has been proposed to explain the decline of *P. cembra* during the past 8000 years (Furrer, 1955). In Switzerland, extensive anthropogenic exploitation of *P. cembra* has furthermore led to fragmented and small stands in the northern periphery of the Swiss Alps (Furrer, 1955). In contrast, *P. cembra* still forms contiguous and large populations in the South and East of the central Alps in Switzerland, where the climate is continental and thus less suitable for *Picea abies* as the primary competitive tree species (Furrer, 1955).

Moreover, *P. cembra* has a distinct dispersal ecology. The wind-pollinated, wingless seeds are largely dispersed by the European nutcracker *(Nucifraga caryocatactes* L.; Mattes, 1990; Tomback *et al.,* 1993). The corvid bird buries groups of five to twelve *P. cembra* seeds as a winter food resource. The number of caches a bird establishes during one season is estimated to several thousand (Mattes, 1990). Since the nutcracker does not recover all caches during winter, many seeds germinate and seedlings establish in suitable microsites (Mattes, 1990). This regeneration often results in multi-stem tree clusters (Tomback *et al.,* 1993). Accordingly, a molecular analysis suggested that spatially aggregated individuals are often genetically related (Rüegg, 2006). Hence, *P. cembra* is a well suited species to study the effects of potential inbreeding, population fragmentation and small population size on the fitness of offspring.

Although genetic studies on the breeding system of *P. cembra* have found that outcrossing was predominant, outcrossing rates were lower than in other pine species (Krutovskii *et al.,* 1995; Lewandowski and Burczyk, 2000; Belokon *et al.,* 2005; Politov *et al.,* 2008). The relevance of these results is, however, difficult to evaluate, as the realized mating system in natural populations as well as the magnitude of early inbreeding depression have not been examined to date.

To better understand the effects of potential inbreeding in natural populations of *P. cembra*, we experimentally and genetically investigated open-pollinated progenies collected in two population types differing in size and in the degree of fragmentation. We

hypothesized higher inbreeding levels and lower fitness performance in offspring from small and fragmented peripheral compared to respective parameters from large and contiguous core populations, which would be especially important in the light of global change and related increasing interspecific competition predicted for *P. cembra* (Casalegno *et al.,* 2010).

The direct comparison between offspring-derived genetic inbreeding values and experimentally assessed fitness of the same progenies will provide insights to the detectability and relevance of inbreeding in small, fragmented populations of a long-lived tree species.

Materials and Methods

Study species and sampling locations

Pinus cembra L. is a monoecious closed-cone pine of the five-needle subsection *Cembrae* (Liston *et al.,* 1999) in the subgenus *Strobus*. Its natural range expands across the European Alps and the Carpathian mountains (Zoller, 1991; Tutin *et al.,* 1993; Fig. 1). In Switzerland, the present distribution of *P. cembra* consists of two rather distinct ranges: while the species covers large and contiguous areas of the timberline ecotone in the continental central Alps, populations in the northern periphery of the Alps are much smaller and more fragmented (Rikli, 1909). These disjunct occurrences are furthermore characterized by lower haplotypic diversity and higher genetic differentiation at chloroplast microsatellites (Gugerli *et al.,* 2009). Based on the genetic structure detected, a common ancestry of the Swiss *P. cembra* populations from a single glacial refugium can be assumed (Gugerli *et al.,* 2009). Hence, we classified our sampling locations into two major population types: large core populations, located in the contiguous range of the central Alps, versus small and fragmented populations in the northern range of the Alps, representing the periphery of the species' distribution range in the European Alps.

Given the generally low level of fruiting in the sampling season 2007, we obtained open-pollinated cones (N=543) from seven *P. cembra* stands, all located in eastern Switzerland: 38 trees could be sampled in four core populations and 16 trees in three peripheral populations (Table 1, Fig. 1). Trees sampled (N=54) were at least 30 m distant from each other, and only tall single-stem trees were considered. Needle samples of seed trees were also collected to assess maternal genotypes.

Cones were kept in humid sand for three months. Once the cones were dried and shattered, seeds were counted. In *Pinus*, a lack of pollination results in the abortion of ovules in the first year of seed development (Sarvas, 1962). Such undeveloped seeds are easy to distinguish from those that were fertilized, due to their small size and scale-like shape. Therefore, we counted fully developed seeds as well as these first-year aborted ovules per cone. The latter were discarded while sets of fully developed seeds were divided in halves per cone. One half was dried at room temperature for laboratory analyses and the other half was subjected to a germination experiment.

Laboratory analyses and seedling parameters

We determined the average weight of dried, fully developed seeds per cone. We then randomly chose five cones from eight maternal trees per stand and excised embryos from three seeds per cone. After removing and discarding the woody outer part of the seeds, we soaked them in distilled water for two hours to allow easy preparation of embryos. The total number of fully developed seeds we had to prepare to obtain 15 developed embryos per mother tree was later used to calculate a proportion value of filled seeds per individual (Table 2, Fig. 2). DNAs from embryos (N=651) and needles were isolated with the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Nuclear microsatellite (nSSR) analysis followed the protocol published by Salzer *et al.* (2009). Locus Pc3 was excluded from the analysis due to difficulties in amplification. Linkage disequilibrium among microsatellite loci was tested with the program FSTAT 2.9.3 (Goudet, 1995). No significances were detected, thus, we included all seven loci in the analysis. All offspring genotypes were cross-checked with the respective maternal genotypes to avoid bias owing to genotyping error and/or null alleles.

Germination experiment, growth trial and seedling performance

Seeds were stored in a peat/sand mixture in plastic bags and subjected to a cold/warm treatment during winter 2007/2008. With appearance of the first radicles in March 2008, seeds were sown in fertilized (4 gr Rhodo fertilizer per litre substrate) peat/wood fibre substrate (4:1) in pots of 12 cm diameter. We evenly spread all seeds that were available per cone in one pot. Pots were repeatedly randomized and transferred from the greenhouse to a nursery after six weeks. We buried the pots in peat substrate, watered them when necessary and covered them with a sun protection mat during the first four weeks, giving 50 % protection. We monitored germination during the vegetation period 2008. Per pot, we counted newly emerged as well as dead seedlings and determined the number of

surviving seedlings in autumn 2008. All pots were maintained in the nursery during winter 2008/2009 without protection. After assessing seedling winter mortality in April 2009, we randomly chose five pots from all maternal trees to examine the following parameters for two randomly chosen seedlings (N=404, 45 families): (1) shoot length, i.e. distance between the first needle circle and the first horizontal root, (2) root length, i.e. distance between the first horizontal root and the root tip, (3) number of buds on apical shoot, and (4) needle length, i.e. length of the longest needle in the first needle circle.

Data analysis

We calculated the fixation index of offspring per population as $F=1 - (H_o/H_e)$ (where H_o and H_e are observed and expected heterozygosity, respectively; Wright, 1951) with FSTAT 2.9.3. To compare this progeny-derived fixation index with the fixation index in adult trees, we additionally estimated F for independent sub-samples of mature *P. cembra* trees from the seven study populations (16 individuals per stand). These individuals had been genotyped at the same nSSR loci (Gugerli et al., unpubl. data).

We calculated fitness values (FVs) on four levels to compare reproductive output and offspring performance between population types: parental seed production, germination success, seedling survival and seedling growth (Table 2).

For seed production and seedling growth, parameters not resulting in proportional data per se, we calculated relative performance coefficients (RPC): values (average value in case of growth data) obtained per cone were divided by the respective maximum (average) value among all cones. Regarding seed production, this allowed us to calculate one fitness value, comprising data on the parental contribution to the offspring, as a multiplicative function of RPC (Table 2).

Germination success per cone was calculated on the basis of fully developed seeds per cone, which had been adjusted for the average percentage of filled seeds (i.e. fully developed seeds containing an embryo) per maternal tree (Table 2, Fig. 2). Seedling survival comprised the seedling survival percentages during the first vegetation period and the first winter season. Regarding growth data, we first investigated correlations between all morphometric parameters measured using Spearman's rank correlations in SPSS 17.0 (SPSS, 2008). Needle length correlated significantly with all other variables and with $r_S>0.2$ (needle–shoot: $r_S=0.248$; needle–number of buds: $r_S=0.316$; needle–seedling length: $r_S=0.410$; $p=0.01$ in all cases). Furthermore, we failed to detect a density effect of seedlings on needle length despite large variation in the number of emerged seedlings per

pot. Consequently, we considered needle length as representative of first-year growth and used only these values in further analyses.

The requirements of normal distribution and homoscedasticity of data were met based on residual distributions after arcsine square root transformation of FVs. This allowed us to apply mixed hierarchical General Linear Models (GLMs), with maternal tree nested within population nested within population type. The latter was determined as fixed effect, maternal tree and population as random effects.

To be able to account for relationships among variables in analyses of variance, we additionally examined correlations among all response variables we investigated. Germination success proved to be positively correlated with average weight of fully developed seeds ($r_{s(core)}$=0.502, P<0.0001, N=387; $r_{s\ (peripheral)}$=0.225, P<0.01, N=135). Seed weight was also positively correlated with seedling survival ($r_{s\ (core)}$=0.327, P<0.0001, N=341; $r_{s(peripheral)}$=0.333, P=0.078, N=29) and first-year growth in both population types ($r_{s(core)}$=0.632, P<0.0001, N=178 ; $r_{s\ (peripheral)}$=0.327, P=0.110, N=25), though only significantly so in core populations. There was no relevant relationship between seedling density and seedling survival, and we failed to detect a trade-off between total number of seeds and seed weight. Accordingly, we included only seed weight as a covariate in the respective GLMs. As the correlations were inconsistent between population types, we additionally incorporated the interaction between covariate and population type into analysis.

Due to the unbalanced sampling design, we applied the recommended restricted maximum likelihood (REML) approach for the examination of variances and calculation of parameter estimates. Analysis was done in JMP 8 (JMP, 1989-2007), and *P*-values for significance tests of differences between the two population types were adjusted using sequential Bonferroni correction (Holm, 1979).

Results

Genetic analyses

We obtained multilocus genotypes of 651 embryos among 42 out of the 54 Swiss stone pine mother trees sampled in seven populations from two different population types. In the peripheral populations, we obtained data for two of the sampled populations only, because we found no more than two embryos among all seeds in stand FL (Table 1).

Mean offspring fixation indices were positive, but significantly deviated from 0 only in populations NA and ZE (Table 1). By contrast, fixation indices of adult trees were all not significantly different from 0, i.e. they did not indicate deviations from Hardy-Weinberg equilibrium.

Parental seed production and offspring performance

Differences in fitness values between the two population types varied among the four levels examined. For parental seed production, the peripheral populations showed significantly lower FVs than the core populations (Table 3, Fig. 3a). Looking separately at all three RPCs that contributed to this FV showed that the differences between population types were large for each of these factors (Table 4). Average total seed set was two times higher in core than in peripheral populations. On average, almost 92 % of seeds were fully developed in core populations, meaning that about 8 % of the total seed set had been aborted during the first year of seed development. By contrast, almost 44 % of ovules had been aborted in peripheral populations. Likewise, the weight of fully developed seeds averaged more than 220 mg in core populations, while the mean seed weight in peripheral populations was less than 170 mg.

The proportion values of filled seeds among fully developed seeds, i.e. the rate of embryo abortion used to adjust the FV germination success, showed differences between population types (Fig. 2). In the core populations, 67.52 % ± 0.03 SE of the fully developed seeds that we fractured contained viable embryos. In contrast, 23.06 % ± 0.03 SE of the fully developed seeds collected in the peripheral populations were filled with embryos, reaching a minimum of 3.93 % ± 0.04 SE in FL.

Regarding seedling performance, germination success was significantly lower in peripheral than in core populations (Table 3, Fig. 3b). On average, 10.09 % ± 0.02 SE of the filled seeds from the peripheral populations germinated, contrasting a germination success of 50.88 % ± 0.02 SE in filled seeds from the core stands. The lowest value was

observed in the peripheral population FL, where no seedling emerged. Furthermore, the influence of the covariate average seed weight in the GLM on germination success was significant, together with the significant interaction between seed weight and population type (Table 3). No significant differences were detectable in seedling survival and first-year growth between population types (Table 3, Fig. 3c, d). The influence of seed weight was significant for both fitness parameters, but not its interaction with population type (Table 3).

Variance components revealed differences in the extent of variation among populations within population types and among maternal trees within populations (Table 3). The latter proved to explain the largest amount of variation regarding parental seed production, but approximated the proportion value explained by population within population type with increasing age of seedling. An increasing proportion of variance was explained by residuals in germination success (58 %) and particularly in seedling survival and growth (> 75 %). This suggested that random effects, which were not included in our model, determined the outcome of these fitness factors to a large degree.

Genetic parameters and seedling performance

We failed to detect significant relationships between population-wide offspring fixation indices and population means of FVs. Regression analysis between F_{IS} values and average seed production per population revealed a very low R^2=0.017 (P=0.807). The relationship between average germination success and population-wide F_{IS} values was also not significant (p=0.143) with R^2=0.386. R^2 between F_{IS} values per population and average seedling survival was 0.386 (p=0.188), while F_{IS} and average seedling growth resulted in R^2=0.241 (p=0.323).

Discussion

The probability of inbreeding is high in natural populations characterized by small population size and fragmentation. Mating among genetically related individuals can lead to a reduction in fitness and might hence hamper the long-term persistence of such populations (Keller and Waller, 2002). We assumed that inbreeding rates are thus elevated in small and fragmented occurrences of *P. cembra*, potentially reducing their offspring fitness. In this study, we combined molecular genetic analyses, i.e. data on inbreeding coefficients, of open-pollinated offspring from large and small *P. cembra* populations with fitness experiments of offspring from the same progenies. We found significant differences regarding parental seed production and germination success between the two population types, while offspring performance did not differ in survival and growth. Additionally, we found striking differences in embryo abortion rates. We took these results as an indication of higher inbreeding depression at early life stages in small and fragmented populations than in large and contiguous populations, as predicted by theory. However, molecular techniques did not allow us to document substantial inbreeding in the offspring since aborted embryos cannot be genotyped readily. Accordingly, we failed to detect a relationship between the positive but low fixation coefficients with experimental fitness data.

No relationship between offspring performance and inbreeding

Seedling performance did not correlate with genetic inbreeding parameters in our study on *P. cembra*, which is per se not surprising in light of the controversially discussed relationship between heterozygosity and fitness (Savolainen and Hedrick, 1995; Britten, 1996; Slate and Pemberton, 2002; Balloux *et al.,* 2004; Pemberton 2004; Chapman *et al.,* 2009; Hansson, 2010). Moreover, it has to be considered that early-acting inbreeding depression might have led to the underestimation of real inbreeding rates (Kärkkäinen and Savolainen, 1993; Husband and Schemske, 1996; Rajora *et al.,* 2002; del Castillo and Trujillo, 2008). Since conifers have one of the highest numbers of lethal equivalents known (Namkoong and Bishir, 1987; Savolainen *et al.,* 1992), an increase in homozygosity is likely to have strong negative effects on offspring. In line with this, inbreeding in conifers has been shown to cause high embryo abortion rates during seed development (Koski, 1971; Charlesworth and Charlesworth, 1987; Kärkkäinen *et al.,* 1999). In our study, around 76 % of the fully developed seeds collected in the peripheral

stands were empty, while seeds from large populations showed on average 30 % embryo abortion. In accordance with other studies on outcrossed conifers (e.g. Mosseler *et al.,* 2000; Rajora *et al.,* 2002), we thus take the high embryo abortion rates as an indication of inbreeding depression at the earliest life stage. At the same time, our results underline that molecular genetic investigations on inbreeding in predominantly outcrossing species - even if derived at an early life stage, i.e. in embryos - will not necessarily elucidate the magnitude of inbreeding. Hence, a significant relationship between inbreeding and offspring performance is difficult to detect, even more so as heterozygosity-deduced inbreeding coefficients based on neutral molecular markers may not be representative of fitness-relevant loci.

Different inbreeding coefficients between life stages

In accordance with the results from other studies on stone pines (Krutovskii *et al.,* 1995; Politov and Krutovskii, 2004; Politov *et al.,* 2006; Bower and Aitken, 2007; Politov *et al.,* 2008), we found significantly positive fixation indices only in progeny arrays and not in adult samples. Such a decrease in heterozygote deficiency with increasing age is considered as an indicator for selection against inbreds during successive life stages in trees (Morgante *et al.,* 1993; Krutovskii *et al.,* 1995; Mitton *et al.,* 1997). Nevertheless, the *F* values measured in our progeny arrays were only slightly positive compared to other studies on conifer offspring (e.g. Morgante *et al.,* 1993; Krakowski *et al.,* 2003; Politov *et al.,* 2006; Bower and Aitken, 2007; -Politov *et al.,* 2008). We suggest that this discrepancy might also result from early-acting inbreeding depression during seed development, leaving only a few inbred embryos for sampling in our study. One should note that our genetic parameters were based on genotypes derived from viable embryos. Hence, they are indicative of tolerance of populations and individuals to inbreeding during seed development, which might differ depending on inbreeding levels and maternal genotypes (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Kärkkäinen *et al.,* 1999).

Lower parental seed production in peripheral populations

Peripheral populations performed significantly worse regarding parental seed production than did core populations. Seed production combines processes that take place over a two-year period in *P. cembra* (Zoller, 1991). Hence, the reproductive cycle is influenced by various abiotic and biotic factors, particularly climatic conditions (Houle and Filion, 1993, and references therein) over an extended period. According to Schnidrig (1935), fruit set

in *P. cembra* depends mainly on the temperature during cone initiation, which suggests that adverse weather conditions might have caused lower total seed numbers in the peripheral compared to the core populations. Furthermore, *P. cembra* trees in the peripheral stands often grow on rocky outcrops, since these offer safe sites from competition by abundant *Picea abies* and are also less prone to grazing. At the same time, soil characteristics along with resource limitation at these sites might cause lower seed weight in peripheral compared to core populations. Supporting the assumption that seed weight covaried with resource availability, we detected a similarly low seed weight in the core population ZE, where trees also grew on rocky ground with shallow soil.

The third parameter examined regarding parental seed production was the rate of unfertilized ovules, which depends on pollination success (Sarvas, 1962). Pollen is wind-dispersed in pines and was shown to travel large distances (Koski, 1970). Therefore, high pollen abundance could be assumed in stands of *P. cembra*. Nevertheless, our peripheral populations showed high rates of unfertilized ovules, indicating pollen limitation. In these small stands, the number of local compatible mates is likely reduced compared to populations with large census sizes. Together with a lower population density, one can assume that total pollen availability is lower and less even in peripheral than in core populations, which in turn increases the likelihood of correlated mating, biparental inbreeding or even selfing. The high genetic differentiation among these peripheral populations (Gugerli *et al.,* 2009) also indicates that gene flow among fragmented stands, which would have the potential to counteract inbreeding, is limited. As numerous factors can affect seed production, we found a large variation in seed production among maternal trees within populations, but even more so between population types in that peripheral populations revealed lower seed production than did core populations.

Germination success, seedling survival and first-year growth

The small peripheral populations showed significantly lower germination success compared to large core populations, while seedling survival and growth did not differ between the two population types. This could partly be due to maternal effects, known to be important for seedling performance (Bishir and Namkoong, 1987; Roach and Wulff, 1987; Wolfe, 1993; Lindgren and Wei, 1994). In line with this, we detected a positive influence of seed weight on germination success, seedling survival and first-year growth, indicating that maternal resource allocation did affect offspring performance in *P. cembra*. Interestingly, the interaction between population type and seed weight was only

significant for germination success, but not for seedling survival and growth. Thus, seed weight influenced seedling emergence to a different degree in core and in peripheral populations. This observation might suggest that germination success does not necessarily increase, once a certain threshold value of seed weight is exceeded. However, it is assumed that maternal effects on seedling performance become less important with increasing seedling age (Wolfe, 1993). Indeed, we found that the variation explained by maternal trees within populations diminished in later life stages. Differences in germination success between the two population types were still significant after accounting for seed weight. Thus, other factors than maternal effects have to be considered as influencing germination in *P. cembra*. In conifers, inbreeding depression is thought to be a major cause for both embryo abortion and reduced performance in subsequent life stages, including germination, seedling survival and growth (e.g. Husband and Schemske, 1996). In line with this, we found significantly lower germination success, but no differences in seedling survival and first-year growth in peripheral compared to core populations. While early life stages are expected to be largely influenced by the expression of highly deleterious alleles, moderately deleterious mutations are thought to have an impact on traits expressed at later life stages, such as vigour and metric traits (Mitchell-Olds and Guries, 1986; Husband and Schemske, 1996; Sorensen, 2001). Thus, the similar performance of progeny from both population types with increasing age could be explained by decreasing inbreeding effects. At the same time, sensitivity of inbreeding depression to environmental conditions has to be considered. Because inbreeding effects tend to be stronger in stressful environments (reviewed in Armbruster and Reed, 2005), negative effects on inbred offspring performance in seedling survival and growth might be more pronounced under natural conditions than in the nursery. However, this could only be investigated by reciprocal transplantation experiments, which were beyond the scope of this study.

Conclusions

The results of this study emphasize the importance of several factors determining reproductive output and potential for regeneration in *P. cembra*: a combination of lower pollen quantity and diversity, adverse weather conditions, resource limitation and higher rates of inbreeding are possible causes that have lead to limited offspring performance in small and fragmented peripheral *P. cembra* populations compared to large and contiguous core populations in the central Alps. This limitation may hamper the long-term persistence of peripheral populations. The on-going changes in climatic conditions are predicted to result in a migration of the species to higher altitudes (Casalegno *et al.,* 2010). In *P. cembra*, migration almost exclusively depends on bird-mediated seed dispersal (Furrer, 1955), in which context reproductive output and the proportion of viable seeds are crucial (Salzer *et al.*, submitted). Taken together, our observations suggest that small and fragmented *P. cembra* populations in the northern periphery of the Swiss Alps are potentially vulnerable owing to uncertain regeneration. Furthermore, our study clearly showed negative consequences of fragmentation and small population size on a long-lived and wind-pollinated tree species, which have formerly been considered to be less susceptible to such effects (Kramer *et al.,* 2008).

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Tables

TABLE 1 Sampling and inbreeding coefficients for offspring and adult *Pinus cembra* trees from core and peripheral populations in the eastern Swiss Alps. Number of families ($N_{families}$), number of cones (N_{cones}) sampled, number of embryos genotyped ($N_{embryos}$) per population and fixation indices (F).

Population		$N_{families}$	N_{cones}	$N_{embryos}$	**Fixation indices**	
					$F_{Offspring}$	F_{Adults}
Core						
ZE	Zernez	10	131	121	0.103*	.074
SM	St Moritz	11	98	121	0.040	0.029
AV	Avers	7	67	105	0.053	0.002
DA	Davos	10	91	120	0.013	).109
Peripheral						
RA	Rautialp	8	82	94	0.068	).056
NA	Neuenalp	5	47	88	0.139*	).128
FL	Flumserberge	3	27	2	--	).124

* significantly different from 0

TABLE 2 Calculation design of fitness values (FV) for Pinus cembra offspring from populations in the eastern Swiss Alps. FVs are functions of relative performance coefficients (RPC) per life stage.

	FV Parental seed production	**FV Germination success**	**FV Seedling survival**	**FV Seedling first-year growth**
RPC included in FV	RPC total number of seeds x RPC number of fully developed seeds x RPC average weight of dried fully developed seed	Total number of emerged seedlings / total number of seeds x average proportion of filled seeds among fully developed seeds per maternal tree	Germination survival x winter survival	RPC average needle length

TABLE 3 Results of mixed hierarchical General Linear Models on parental seed production and early life stage fitness in *Pinus cembra* offspring from core and peripheral populations in the eastern Swiss Alps. *F*-values and *P*-values are given for population type, covariate and their interaction and variance components for the random effects population nested within population type, maternal trees nested within population nested within population type and residuals.

Source of variation	***F***	***P***	**Variance component %**		
			Population [population type]	**Maternal tree [[population] population type]**	**Residuals**
Parental seed production					
Population type	13.22	0.0126*	24.94	39.12	35.94
Germination success					
Population type	17.15	0.0083*	18.29	22.96	58.75
Covariate seed weight	12.24	0.0005*			
Seed weight x population type	6.29	0.0125*			
Seedling survival					
Population type	0.63	0.4538	9.81	10.83	79.36
Covariate seed weight	11.71	0.0007*			
Seed weight x population type	0.25	0.6157			
Seedling first-year growth					
Population type	0.00	0.9845	12.08	12.45	75.48
Covariate seed weight	13.38	0.0004*			
Seed weight x population type	0.15	0.6947			

TABLE 4 Seed production traits estimated for cones collected from *Pinus cembra* from core and peripheral populations in the eastern Swiss Alps. Average values per population type are given in bold. Total number of seeds, proportion of unfertilized aborted ovules, number of fully developed seeds and average weight per dried fully developed seed are given. Values are means among maternal progenies, with standard deviation in parentheses.

Population	**Total number of seeds**	**Percent of aborted ovules**	**Number of fully developed seeds**	**Average weight per fully developed seed [g]**
Core	**55.9 (30.1)**	**8.53 % (14.29)**	**51.1 (29.4)**	**0.225 (0.074)**
Zernez	79.5 (29.6)	8.77 % (13.81)	72.8 (30.3)	0.162 (0.048)
St Moritz	37.5 (19.9)	14.09 % (17.98)	31.1 (16.2)	0.245 (0.076)
Avers	60.5 (22.1)	1.22 % (2.35)	59.8 (22.0)	0.261 (0.055)
Davos	38.5 (18.0)	7.59 % (1.32)	35.1 (17.5)	0.268 (0.049)
Peripheral	**27.1 (21.1)**	**43.71 % (37.05)**	**13.0 (14.7)**	**0.167 (0.065)**
Rautialp	24.6 (14.1)	34.81 % (36.26)	16.2 (15.4)	0.179 (0.070)
Neuenalp	45.0 (24.6)	67.72 % (26.43)	13.6 (15.0)	0.147 (0.062)
Flumserberge	3.6 (2.9)	26.38 % (35.76)	2.4 (1.9)	0.164 (0.033)

Figures

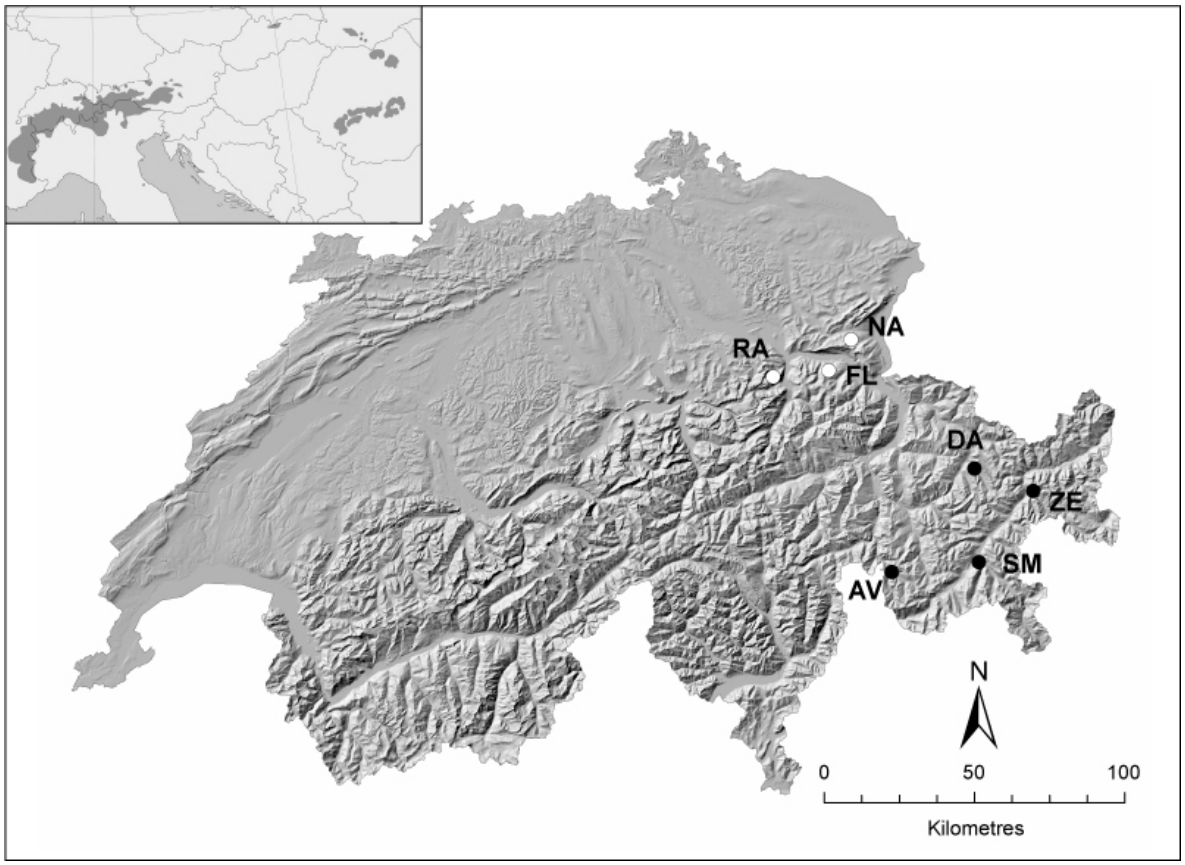


FIG. 1 Locations of the *Pinus cembra* populations sampled in the Swiss Alps (for abbreviations see Table 1). Open circles indicate peripheral populations and filled circles display core populations. Grey areas in the inset illustrate the species' natural distribution range

(distribution map courtesy to Euforgen: http://www.bioversityinternational.com/Networks/Euforgen).

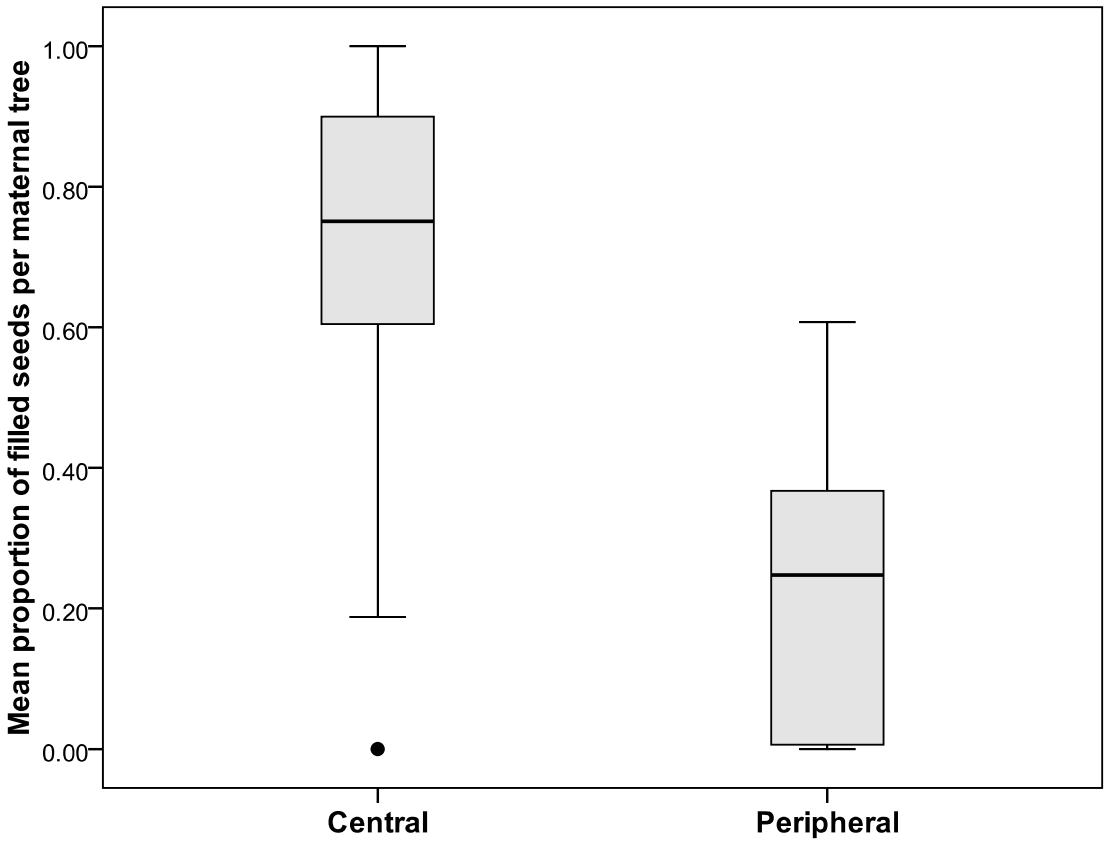
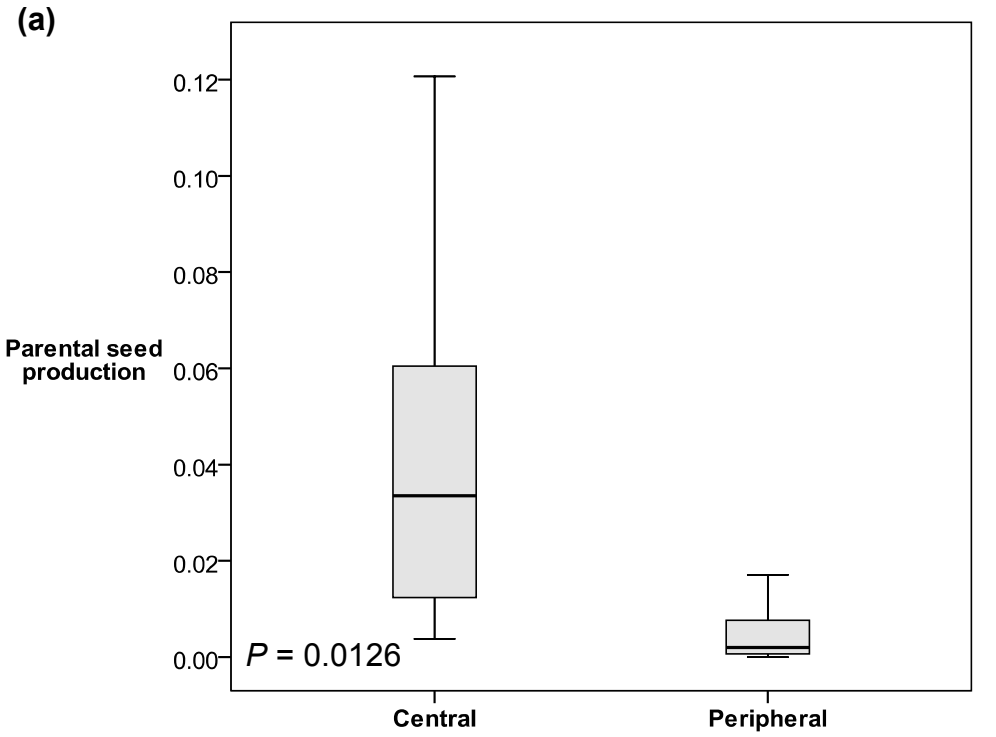
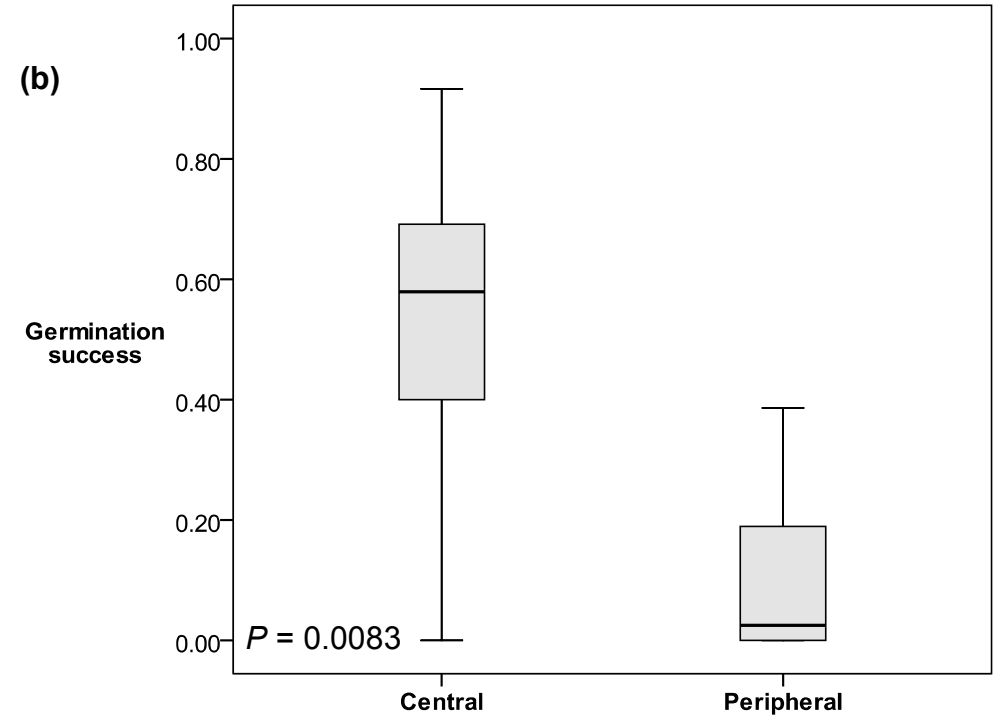


FIG. 2 Boxplots showing the mean proportion of filled among fully developed seeds, calculated for maternal trees from core and peripheral populations of *Pinus cembra* in the eastern Swiss Alps.


(a)
Parental seed production
0.12
0.10
0.08
0.06
0.04
0.02
0.00
P = 0.0126
Central
Peripheral


(b)
Germination success
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.0083
Central
Peripheral

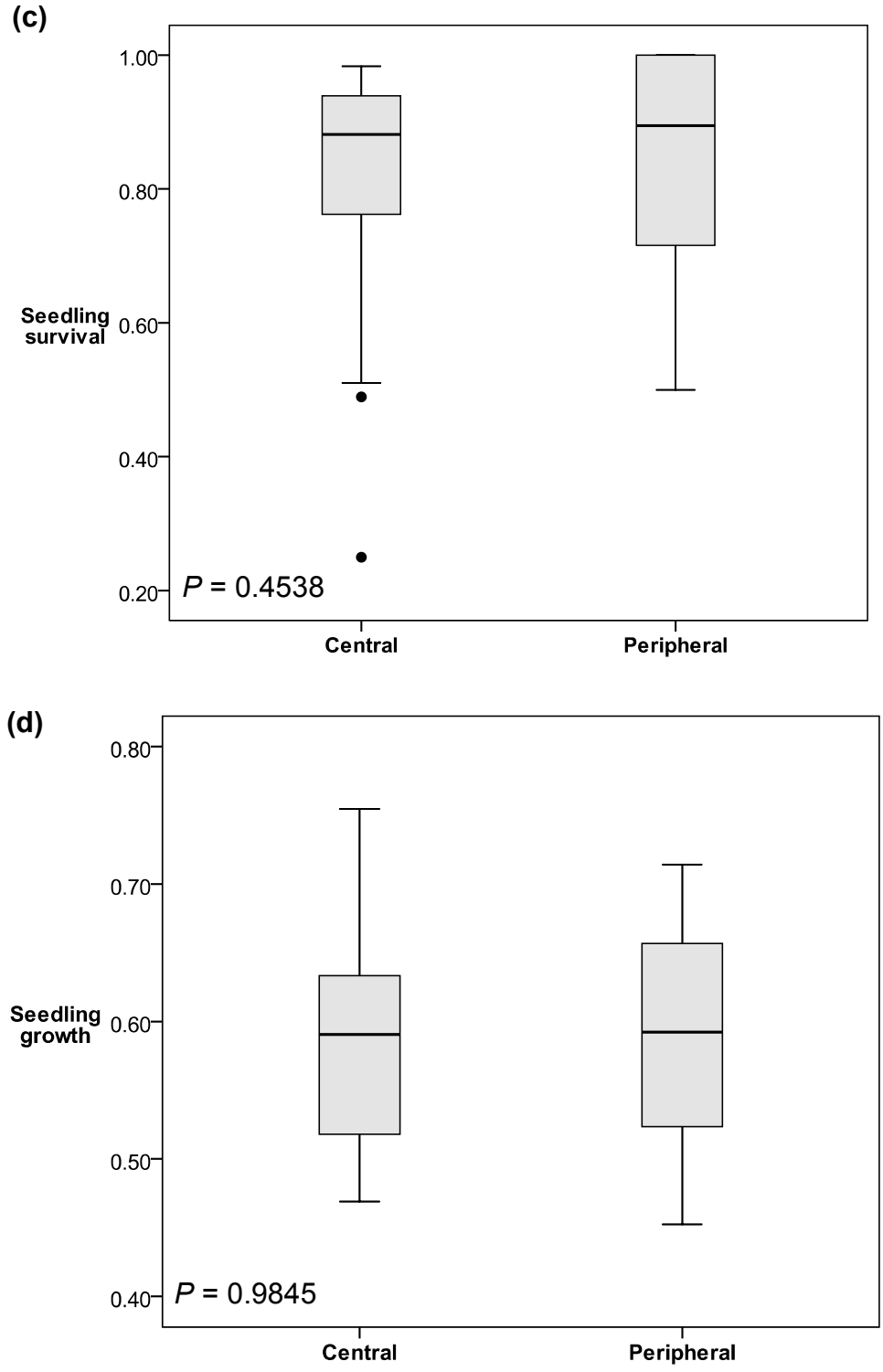


FIG. 3 Boxplots showing mean performance at four life stages, calculated for maternal trees from core and peripheral populations of *Pinus cembra* in the eastern Swiss Alps. Graph (a) displays parental seed production, (b) germination success, (c) seedling survival and (d) seedling first-year growth. *P*-values refer to GLMs (see Materials and Methods section).

Chapter IV

Isolation and characterization of polymorphic nuclear microsatellite loci in *Pinus cembra* L.

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Abstract

We developed eight polymorphic nuclear microsatellite markers for the Swiss Stone Pine (*Pinus cembra* L.), of which seven may be amplified in a multiplex PCR reaction. Allelic polymorphism across all loci and 40 individuals representing two populations in the Swiss Alps was high (mean=7.6 alleles). No significant linkage disequlibrium was displayed between pairs of loci. Significant deviation from HWE was revealed at three loci in one population. Cross–amplification was achieved in two related species within the genus (*P. sibirica* and *P. pumila).* Thus, the markers may be useful for population genetic studies in these three pine species. They will be applied in ongoing projects on genetic diversity and patterns of gene flow in *Pinus cembra*.

Swiss Stone Pine (*Pinus cembra* L., Pinaceae) is a closed–cone conifer species of the section *Strobus*. Its natural distribution comprises the European Alps and the Carpathian mountains. The monoecious trees occur in altitudes between 1000 and 2850 m above sea level, the female flowers are wind–pollinated and the seeds are actively dispersed by the European Nutcracker (*Nucifraga caryocatactes* L.) (Mattes, 1990; Zoller, 1991). Chronic deforestation and land use change have resulted in the fragmentation and deterioration of Swiss Stone Pine populations (Rikli, 1909). Thus, detailed information on genetic diversity, pollination biology and gene flow in this species will be highly valuable for its future conservation and management.

Microsatellites (simple sequence repeats, SSRs) proved to be very useful for such studies (e.g. Aldrich *et al.,* 1998; Marquardt and Epperson, 2004; Robledo-Arnuncio and Gíl, 2005). As nuclear (n) SSRs have not yet been available for Swiss Stone Pine and transfer from other pine species is prone to failure (González-Martínez *et al.,* 2004), we aimed to isolate a species–specific set of nSSRs from an enriched genomic library. Due to the conifers' very large and highly repetitive genome (Wakamiya *et al.,* 1993; Kinlaw and Neale, 1997; Murray, 1998), the development of single polymorphic SSRs requires a much higher effort than in species with a simpler genome. In order to improve the cost–benefit value, we therefore combined the *de novo* development of markers for *Pinus cembra* with their additional transfer to closely related species.

For the library enrichment, we followed the protocol of (Edwards *et al.,* 1996). DNA from embryos of *P. cembra* was isolated with the DNeasy 96 Plant Kit (QIAGEN) following the manufacturer's protocol. The DNA from three seeds of different individuals was pooled (ca 5 µg), digested with the *Rsa*I enzyme, ligated to the *Mlu*I adaptor (5'–CTCTTGCTTACGCGTGGACTA–3') and amplified through PCR. Amplified PCR products were hybridized twice with di– (GA, GT, AT, GC), tri– (CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) repeats bounded to Hybond N^+ filters (GE Healthcare). Enriched fragments were then amplified and cloned using the TOPO TA Cloning Kit (Invitrogen). From three libraries, 624 randomly chosen clones were sequenced in the forward direction with the M13 universal primer, using the DYEnamic ET Terminators Sequencing Kit (GE Healthcare) and run on a GE Healthcare MegaBace 1000 automatic sequencer. Microsatellite motifs were detected in 265 (42.5 %) of the sequences, of which 226 clones contained microsatellite stretches that were either too short, too long or too close to the vector for primer designing, or they revealed sequences that were homologous among each other. We sequenced the reverse direction of the

remaining 39 clones and designed primer pairs in the flanking regions of the microsatellites (Primer3 software, Rozen and Skaletsky, 1999). After screening twelve individuals from three populations, eight out of the 39 primers displayed consistent and polymorphic patterns, whereas the others were discarded due to amplification failure, multi–banding patterns, excessive stuttering or monomorphic structure. The selected primer pairs amplify seven di– and one trinucleotide repeats (Table 1).

To characterize these eight SSR markers, we analysed 40 trees in two geographically distant populations sampled in Switzerland (Rautialp and Engstlenalp; 20 individuals per population). Genomic DNA was isolated from dried needles using the QIAGEN DNeasy 96 Plant Kit. Amplification reactions of 10 μL contained ca 20 ng of template DNA, 1×PCR Multiplex Mix (QIAGEN), and 0.2, 0.4 or 0.6 μM of each primer (Table 1). Two PCR profiles were employed to successfully amplify the microsatellite loci: 94 ºC for 15 min, followed by 30 cycles of denaturation at 94 ºC (30 s), annealing at 54 ºC (multiplex) or 62 ºC (singleplex) for 30 s, extension at 72 ºC (50s) and final extension at 72 ºC for 15 min (Table 1). Amplification reactions were carried out in a PTC 100 cycler (Bio–Rad Laboratories). Fluorescence–labelled amplicons from the two PCRs were pooled and run on an ABI 3130 automated sequencer (Applied Biosystems). Alleles were sized according to an internal size standard (LIZ, Applied Biosystems) using Genemapper 3.7 (Applied Biosystems).

Standard genetic diversity parameters and deviation from Hardy–Weinberg equilibrium (HWE) were estimated using Genepop 4.0 (Rousset, 2008). FSTAT 2.9.3.2. (Goudet, 2002) was applied to check for linkage disequilibrium between pairs of loci.

The number of alleles per locus ranged from two to 17 in the 40 individuals genotyped, with a total of 61 alleles (Table 1). No significant linkage disequilibrium was observed between pairs of loci (data not shown; $P>0.05$ in all cases, after Bonferroni correction). Observed and expected heterozygosities are listed in Table 1. Significant deviations from HWE were displayed by loci Pc3, Pc7 and Pc18 in Engstlenalp. Null alleles could be expected at these loci, but it should be considered that both populations were relatively small, and, thus, the deficit of heterozygotes could be due to small effective population size.

Segregation analyses were conducted with DNAs from twelve *P. cembra* embryos, obtained from one cone of a source tree with a known genotype. For all loci, either heterozygous or homozygous in the mother tree, at least one of the maternal alleles could

be identified in each seed genotype. These findings are in accordance with Mendelian inheritance.

To check for the transferability of the eight newly developed markers to closely related species, we analysed samples from *P. sibirica* and *P. pumila* (twelve samples from one population each). The two congeneric species share many morphological and life–history traits, but *P. pumila* was recently considered as more distantly related to *P. cembra* than was *P. sibirica* (Liston *et al.,* 1999). For *P. sibirica*, six markers displayed good amplification. All six loci were polymorphic, with 2–9 alleles per locus and a total of 31 alleles scored. For *P. pumila*, only three markers showed a scorable and polymorphic pattern (5–12 alleles per locus and a total of 26 alleles). One additional primer pair displayed good amplification but was monomorphic in the set of twelve individuals tested. These different rates of transferability may reflect the phylogenetic relationships among the three Stone pine species (Liston *et al.,* 1999). Numbers of alleles, allele size range, expected and observed heterozygosities are listed in Table 2. Loci Pc3 and Pc22 showed significant deviations from HWE in the *P. pumila* samples, so null allele occurrence cannot be excluded. No significant linkage disequilibrium was detected between locus pairs in *P. sibirica* and *P. pumila*, respectively (data not shown; $P>0.05$ in all cases, after Bonferroni correction).

These eight nuclear loci should be very useful for diversity and population genetic studies of the species. Characterized by high polymorphism (probability of exclusion >99 %), they may be very efficient for forthcoming parentage analysis and monitoring gene flow in *P. cembra*. Furthermore, the cross–species amplification indicated the suitability of these markers for population genetic studies in related pine species.

Acknowledgements This study was funded by grants from the Swiss National Science Foundation (SNF No. 3100AO–113918) and the EC–supported Network of Excellence EVOLTREE (No. 016322).

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Tables

TABLE 1. Characteristics of eight microsatellite loci isolated for the Swiss Stone Pine (*Pinus cembra*): GenBank accession number, primer sequences, primer labelling (fluorescent dye), repeat motif and copy number of the sequenced clones, primer concentration, fragment size range and total number of alleles (N_a) scored in 40 *P. cembra* individuals from two populations. Genetic diversity estimates for two *Pinus cembra* populations (Rautialp and Engstlenalp, Switzerland; 20 individuals each): N_A = number of alleles; H_o= observed heterozygosity, H_e = expected heterozygosity; HWE = test for Hardy–Weinberg equilibrium (probability test, Rousset 2008): n.s. = not significant ($P > 0.05$), $P < 0.05$ significant (*), $P < 0.001$ highly significant deviation from HWE (**), n.a. = not applicable (one of the two alleles was represented by only one copy).

Locus name (Accession no.)	**Primer sequence 5'–3'**	**Dye (forw. primer)**	**Repeat motif**	**Primer conc. (µM)**	**Allele size range (bp)**	N_a	**Rautialp**				**Engstlenalp**			
							N_A	H_o	H_e	HWE	N_A	H_o	H_e	HWE
Pc 1b (EU629205)	**F:** CCACCATCTTGTTTTTGTGTTC **R:** TTCTCTCCACCCAGCCTAAA	FAM	$(GT)_{19}$	0.2	166–202	10	6	0.90	0.76	n.s.	9	0.80	0.85	n.s.
Pc 3 (EU629208)	**F:** AGGGACTTCAAGGGAGTCAT **R:** CTTGGTAACCCCAAGGGACT	PET	$(CT)_9$ $(AT)_5$	0.6	195–269	17	12	0.80	0.87	n.s.	8	0.35	0.42	0.006 *
Pc 7 (EU629210)	**F:** TGGTCATGTTTCCTTATCAATTC **R:** TCGCAAACCATCTATTGACC	FAM	$(CT)_6$ $(AT)_4$ $(GT)_{25}$	0.2	342–384	7	6	0.35	0.53	n.s.	7	0.30	0.63	0.000 *
Pc 18 (EU629204)	**F:** TTCCCAAAGACCATAGAACCA **R:** TCATGAAATATTACGTCCCTTATCC	NED	$(TG)_{12}$	0.2	152–156	3	2	0.40	0.05	n.a.	3	0.20	0.14	0.026 *

TABLE 1 Continuation

Locus name (Accession no.)	Primer sequence 5'–3'	Dye (forw. primer)	Repeat motif	Primer conc. (µM)	Allele size range (bp)	N_a	Rautialp				Engstlenalp			
							N_A	H_o	H_e	HWE	N_A	H_o	H_e	HWE
Pc 22 (EU629203)	**F:** TGTCCCCAGATGTAGTATAATCAA **R:** GGTTCAACCCACCCATTCT	PET	$(CA)_7TA(CA)_{12}(TA)_2$	0.4	345–375	5	3	0.45	0.49	n.s.	5	0.50	0.51	n.s.
Pc 23 (EU629206)	**F:** GGGCATCATTATTTTCTTACAA **R:** CTTGATATACCATGCCACAACC	VIC	$(TG)_6CG(TG)_2$	0.2	221–257	12	9	0.90	0.82	n.s.	10	0.80	0.85	n.s.
Pc 25 * (EU629207)	**F:** CGGTGCAAGATGTTGTCCT **R:** GTGAGATCTAGGAAGAACTATATACCG	VIC	$(TC)_{20}(TG)_4$	0.2	128–138	5	5	0.70	0.64	n.s.	5	0.60	0.57	n.s.
Pc 35 (EU629209)	**F:** CCCCTCGATTCGAAAATGAT **R:** TTGGAATGTTGCAGTCCTGA	PET	$(GTT)_7$	0.2	162–165	2	2	0.50	0.05	n.a.	1	–	–	–

*marker amplified in singleplex PCR owing to higher annealing temperature.

TABLE 2. Genetic diversity estimates for *P. sibirica* and *P. pumila* (12 individuals each), using cross-amplified microsatellite markers isolated for *P. cembra*: N_A=number of alleles, allele size range, H_o=observed heterozygosity, H_e=expected heterozygosity, HWE=test for Hardy–Weinberg equilibrium (probability test, Rousset, 2008): n.s.=not significant (P>0.05), P<0.05 significant (*), P<0.001 highly significant deviation from HWE (**). Note that only those markers are listed which were successfully cross-amplified.

	N_A	Allele size range (bp)	H_o	H_e	HWE
P. sibirica					
Pc 1b	9	174–212	0.92	0.83	n.s.
Pc 7	7	354–378	0.83	0.79	n.s.
Pc 18	4	152–158	0.75	0.74	n.s.
Pc 22	5	342–354	0.91	0.76	n.s.
Pc 23	4	205–235	0.67	0.61	n.s.
Pc 35	2	152–162	0.17	0.16	n.s.
P. pumila					
Pc 3	8	209–295	0.25	0.80	0.000**
Pc 18	1	144	–	–	–
Pc 22	5	318–376	0.58	0.78	0.036*
Pc 23	12	215–291	0.83	0.91	n.s.

Synthesis and Conclusions

The aim of this study was to elucidate the two-step gene flow system of *Pinus cembra*, comprising wind pollination and subsequent seed dispersal through the European nutcracker. The investigation of the spatial genetic structure within one study population revealed the consequences of this gene flow system on the genetic structure within a small, peripheral *P. cembra* stand, including high probabilities for inbreeding. Subsequently, the potential effects of elevated inbreeding on early life stages in *P. cembra* offspring, sampled in two population types differing in size and degree of fragmentation, were investigated.

Below, I view the manuscripts in hand in the context of the entire thesis with respect to ecological relevance, finishing with some general conclusions. In an outlook discussing potential limitations of the study, I will describe future research topics that might complement the present work.

New insights regarding gene flow in *P. cembra* – effects of the relationship between Swiss stone pine and the European nutcracker

The biology of *P. cembra* is closely linked with the behaviour of the European nutcracker (*Nucifraga caryocatactes*) (Mattes, 1990). In past centuries, this bird was considered a major threat for the persistence of Swiss stone pine populations, since its excessive foraging on pine seeds was believed to impede the tree's regeneration (Hess, 1916). During the early 20th century, however, the role of the birds' caching behaviour for the rejuvenation of this pine species was increasingly discussed and subsequently valued (Campell, 1950). Since not all seeds which the nutcrackers have cached as winter food are recovered, the bird strongly impacts the regeneration capacity of the pine and, in particular, its ability to colonize new habitats. Thus, the nutcracker can be considered the main vector for both short- and long-distance gene flow via seed. Nevertheless, only little has been known to date about the influence of this mutualistic relationship on the genetic structure of *P. cembra* populations.

Considering that the nutcracker is a far-ranging bird (Mattes, 1990), gene flow could be expected to be random in *P. cembra*, even more so since pollen is previously dispersed by wind (Latta and Mitton, 1997, 1999; Richardson *et al.,* 2002). In line with this, molecular genetic results on trees in the study stand Rautialp revealed that both wind

and birds facilitate gene dispersal across long distances (*Chapter I*). Furthermore, the birds assist in an altitudinal upwards shift of the tree species, since juvenile trees were found beyond the upper altitudinal occurrence of adult trees. Nonetheless, data also indicate that seeds are collected in a kin-structured manner (cf. Furnier *et al.,* 1987) and are additionally spread within a spatially restricted area per caching event, which is in line with optimal foraging theory (Schoener, 1971). Hence, clusters of related stone pine individuals may establish, especially in areas where low adult tree density reduces an overlap of seed shadows (*Chapter II*). Since this non-random distribution of genotypes was found to persist until the mature life stage, the probability of correlated matings, i.e. inbreeding, is subsequently elevated. Inbreeding becomes even more likely considering that pollination distance decreased with increasing proximity between potential mates (*Chapter I*). Accordingly, the spatial distribution of paternal alleles was also found to be non-random (*Chapter II*).

Prior to generalizing these findings, however, it has to be considered that gene dispersal and, hence, the extent of spatial genetic structure (SGS) can be influenced by several factors. Populations in the core of the species' range comprise much higher numbers of potential mates (also through higher connectivity; see Gugerli *et al.,* 2009) and gene flow can consequently be expected to be less restricted. They are furthermore characterized by higher adult tree density (Rikli, 1909; Mattes, 1990), which would promote a less pronounced or even a random SGS. Therefore, probabilities for inbreeding would be reduced. Supporting these hypotheses, inbreeding depression at the earliest life stages – measured as higher embryo abortion and lower germination success – was significantly lower in progenies from large core than in those from small peripheral populations (*Chapter III*). Thus, our findings confirm the general assumption that small populations are prone to the occurrence of inbreeding and genetic drift, with negative effects on offspring (Ellstrand and Elam, 1993; Keller and Waller, 2002; Aguilar *et al.,* 2008). At the same time, the high embryo abortion rates underline that molecular genetic investigations in predominantly outcrossing species – even if derived at the seed stage – will not necessarily elucidate the magnitude of inbreeding (e.g. Rajora *et al.,* 2002; Balloux *et al.,* 2004), even more so of inbreeding depression. Results of our common garden experiment also showed that negative effects of inbreeding decrease with age (Husband and Schemske, 1996), since no significant differences could be detected in the performance of seedlings after germination (*Chapter III*). However, inbreeding depression is known to be sensitive to environmental conditions (Armbruster and Reed, 2005). Thus,

benign settings in the garden might have masked detrimental effects of moderately deleterious alleles, which may, hence, be expressed to a larger extent under natural conditions. This is supported by the results from Rautialp, where inbreeding coefficients were higher within the juvenile than within the adult generation, suggesting that inbred *P. cembra* individuals are selected against during the trees´ successive life stages under natural conditions (Mitton *et al.,* 1997).

Thus, the main findings and conclusions of this study can be summarized as follows:

Conclusion 1: Pollen and seed dispersal occurs over large distances, specifically so across the entire *P. cembra* study population. *(Chapter I)*

Conclusion 2: The nutcracker collects and distributes seeds in a kin-structured way, favouring the establishment of SGS within natural regeneration – especially in areas with low adult tree density. *(Chapter II)*

Conclusion 3: The SGS persists until the mature life stage, thus promoting the occurrence of inbreeding. (Chapter II)

Conclusion 4: Pollination distance decreases with increased tree density, which further increases probabilities for mating among related individuals occurring in spatial proximity. *(Chapter I and II)*

Conclusion 5: Inbreeding negatively affects natural regeneration in *P. cembra*, since it causes higher embryo abortion and lower germination success. At the same time, the magnitude of inbreeding depression at the early life stage cannot necessarily be determined using exclusively molecular genetic markers. *(Chapter III)*

Conclusion 6: Selection against inbred individuals seems to continue during later life stages in *P. cembra*. *(Chapter III)*

Conclusion 7: Inbreeding depression at early life-stages was significantly lower in large and continuous than in small and peripheral populations, supporting the assumption of restricted gene flow and hence, the presence of SGS in the latter. *(Chapter III)*

To conclude, the outcomes of this study suggest that, according to our expectations, small and peripheral *P. cembra* populations are vulnerable to negative effects of small population size and related restricted gene flow – regardless of gene dispersal through far–ranging vectors. Hence, I emphasize that these small populations in the peripheries of the

species' range seem to be especially susceptible to the predicted changes in habitat suitability and interspecific competition (Casalegno *et al.,* 2010).

A sufficiently large reproductive output will be crucial for the persistence of *P. cembra* populations, since this is fundamental for a stable abundance of seed caching birds and, related with that, for the successful establishment of sufficiently high numbers of seedlings despite inbreeding depression. Stable abundances of nutcrackers are furthermore relevant to facilitate an altitudinal upward shift of *P. cembra*, which might become essential in the light of ongoing climate change.

Based on the results of this thesis, I consider that the long-term maintenance of small and peripheral populations asks for special attention. Hence, addressing questions on the effects of reproductive output, individual bird behaviour or genotype x habitat interactions will greatly increase our knowledge about *P. cembra* and will also be very important to sketch the future persistence of its natural populations. Suggestions for future research approaches will therefore be presented in the following chapter.

Outlook

This study shows that a combination of low effective population size and low tree density with directional and kin-structured seed dispersal results in the establishment of SGS. An additional decrease in reproductive output due to adverse climatic conditions, along with limited mate availability and clustering of related individuals, then likely increases inbreeding. At the same time, this can be expected to negatively influence bird abundance and, hence, caching activities, since nutcrackers mainly collect viable seeds (Mattes, 1990). Consequently, negative effects on regeneration and, hence, also on the potential for altitudinal migration of *P. cembra* can be expected. Nevertheless, it has to be noted that these parameters are temporally and spatially variable. Mast years are known to occur in *P. cembra*, increasing probabilities for successful regeneration (Mattes, 1990). Thus, investigation of gene flow processes in relation to reproductive output of trees would be interesting. Nevertheless, such a study would be difficult to realize, since relocation of seed caches has proved extremely challenging (K. Salzer, personal observation). In line with this, the drawback of studies on established seedlings like the present work is that exact determination of seedlings' age is critical. Consequently, no exact conclusions can be drawn on different extents of seed dispersal at the level of cohorts and, thus, among years.

Another interesting research question could be related to bird-mediated seed transport across long distances. Nutcrackers have been observed to carry seeds over more than ten kilometres (Mattes, 1990), suggesting that some gene exchange among small populations still occurs. Considering that even rare gene immigration is expected to decrease a population's vulnerability to genetic drift, more research would be valuable to elucidate gene flow patterns within and among *P. cembra* populations. In the present study, we could not determine the origin of seed and pollen introduced into Rautialp. The ongoing molecular genetic characterization of *P. cembra* stands across the entire Swiss Alps (F. Gugerli, unpublished data) will thus help to detect potential gene flow among populations. Additionally, increasing the haplotypic resolution through genotyping more than four chloroplast microsatellite markers, might reveal a more accurate pattern of the spatial distribution of paternal alleles, i.e. wind pollination processes, among and within populations. For example, the high

frequency of one cpSSR haplotype, abundant in most Swiss populations (Gugerli *et al.*, 2009), likely masked SGS of paternal alleles in Rautialp.

A similar investigation of SGS within other small and peripheral in comparison to large and continuous *P. cembra* stands would provide further information on gene flow and bird behaviour within the two population types. However, such research will be very time- and labour-intensive, since exhaustive sampling of potential parents and regeneration is required, unless probabilistic statistical approaches with respective uncertainties, such as TwoGener, are employed (Smouse and Sork, 2004).

I consider the detailed investigation of abiotic (climatic and soil conditions, influence and distribution of habitats suitable for regeneration) as well as biotic parameters (tree density, cattle and goat grazing, fluctuations in bird abundance and/or individual bird behaviour) as an important issue for future research, since these factors can be expected to influence both mating patterns and patterns of seedling establishment. Similarly, genotype x habitat interactions might play an important role, yet remain unknown in the present work due to the neutral character of microsatellite loci. Hence, the application of markers under selection might prove beneficial in investigating such questions and are currently under study (F. Gugerli *et al.*, unpublished data).

Regarding the inbreeding effects in *P. cembra* discussed above, it has to be noted that the respective study is based on offspring collected in one year only, and replicates per population type and population were restricted due to low seed yield in autumn 2007. Since tolerance to inbreeding effects might differ among individuals and populations (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Hedrick *et al.,* 1999), a repetition and/or an extension of this study to additional populations could be highly useful to substantiate the present results. This becomes also relevant considering that several factors likely affect mating system and, hence, reproductive output, such as weather conditions and/or individual phenology (reviewed by Mitton, 1992).

Previous studies on the relationship between snow cover duration and seedling mortality through infestation with the fungal pathogen *Gremmeniella abietina* showed that trees "in poor condition" are more susceptible: a total of 59.8% of *P. cembra* were killed by *G. abietina* during the first 20 years after planting in an experimental afforestation in the Swiss Alps (Senn, 1999). Thus, duration of snow cover plays an

important role for successful regeneration, and at the same time, it can be expected to be altered with ongoing climate change. Hence, it would be interesting to investigate a potential correlation between the level of inbreeding and seedling mortality through *G. abietina*, also in this case associating variation in adaptive loci to the patterns observed.

Given that inbreeding depression likely also affects later life stages (Husband and Schemske, 1996), it would be interesting to monitor the performance of seedlings from both experiments – those sampled in Rautialp and those sown in the common garden – within the coming years. However, it has to be considered that performance of seedlings in the garden might also be influenced by environmental conditions: progenies collected in different altitudes and climatic conditions might behave differently under abiotic conditions in the common garden in Birmensdorf, masking effects purely associated to inbreeding. This aspect also becomes important considering the sensitivity of inbreeding effects to environmental conditions (Armbruster and Reed, 2005). Nevertheless, such effects could only be minimized through reciprocal transplantation experiments. Ideally, such monitoring of offspring should be conducted until trees reach the mature age, since inbreeding effects can be expected to influence the reproductive potential of individuals (Husband and Schemske, 1996). However, the long generation time of *P. cembra* makes such an investigation difficult.

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